Endothelial progenitor cell dysfunction in diabetes mellitus

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Endothelial Progenitor Cell dysfunction in Diabetes Mellitus

door Cindy J.M. Loomans

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It was men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy their mind

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

So oft in theologic wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!

John Godfrey Saxe (1816-1887)

In memory of my Mom and dedicated to my Dad.

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Chapter 1

General Introduction

Cindy J.M. Loomans

1.1 Endothelial cells and vascular function.

The vascular endothelium is a one cell-layer thick lining of endothelial cells (EC) in all blood- and lymphatic vessels in the body and represents a dynamic border between blood and surrounding tissue. Besides being a physiological barrier, regulating transfer of several molecules and cells, the endothelium also produces a variety of factors that control vascular tone. These regulatory factors, such as nitric oxide (NO), endothelin-1 and prostaglandins, have either vasodilating or vasoconstricting properties[1]. Under normal physiological conditions the monolayer provides a non-adhesive, uninterrupted surface for circulating platelets and leukocytes. However, inflammation of the endothelium caused by several stimuli such as hyperglycemia, hyperlipidemia and other circulating cardiovascular risk factors lead to EC activation. A cascade of events is ignited by EC activation such as upregulation of cell adhesion molecules, adhesion of leukocytes to the endothelial cell lining and trans-endothelial migration of mononuclear cells. These events are also associated with EC apoptosis and disruption of the EC layer; further increasing endothelial dysfunction and the risk of cardiovascular disease[2] (figure 1).

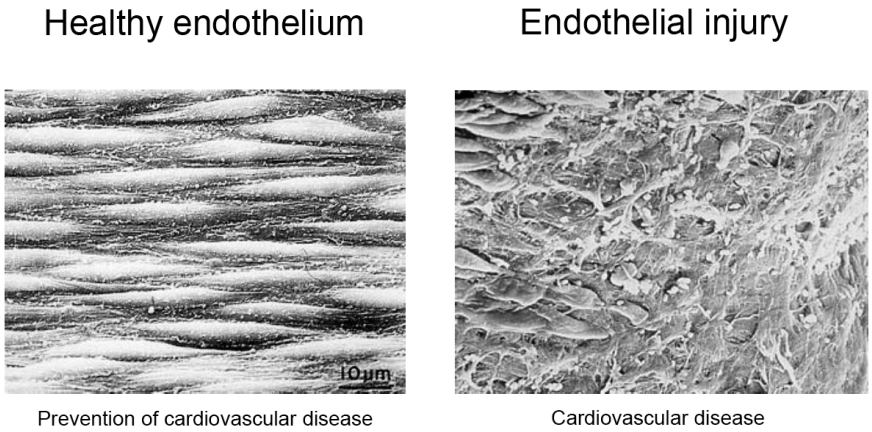

Figure 1 Activated endothelium displays different characteristics, contributing to cardiovascular disease.

Healthy endothelium forms a smooth monolayer of elongated EC, with out adhesive capacities, whereas, injured and inflamed endothelium is disrupted and forms an adhesive surface to all blood cells and platelets. Healthy endothelium protects against cardiovascular disease.

1.2 Postnatal neovascularization

Postnatally, the development and maintenance of the vascular system requires constant remodeling and dynamic adaptation of vessel and network structures in response to functional needs[3]. For this so-called angioadaptation, the formation of new blood vessels (neovascularization) is crucial and the process involves angiogenesis and arteriogenesis[4]. Recently, it was shown that bone marrow-derived cells are also involved in neovascularisation in a process called vasculogenesis[5].

1.2.1. Angiogenesis

Angiogenesis describes the formation of new capillaries from already existing capillaries. It is a physiological process required postnatally for non-pathological processes like endometrial remodeling during the menstrual cycle and wound healing. It also plays a role in pathological processes such as tumor growth, inflammation and rheumatoid arthritis. Angiogenesis is a complex and not yet fully understood process that is tightly controlled by over 20 activators and a similar number of inhibitors. Hypoxia is a driving force for angiogenesis. When an oxygen consuming tissue is deprived of oxygen there is a high need for an adequate blood supply. Hypoxic tissues releases molecules, such as vascular endothelial growth factor (VEGF), that trigger the angiogenic response. VEGF production is stimulated by binding of hypoxia inducible factor-1alpha (HIF-1 alpha) to the hypoxia response area in the VEGF gene promoter region. Many of the angiogenic stimuli promote proliferation, migration of EC and inhibit apoptosis and VEGF is one of these stimuli that mediate these crucial processes of angiogenesis. VEGF activates EC to produce nitric oxide (NO), which mediates vasodilatation and VEGF can also stimulate the release of proteolytic enzymes to dissolve basement membrane surrounding parent vessels creating an environment supporting EC migration and VEGF-receptor mediated proliferation of EC into the tissue. New vessels further mature by production of extra cellular matrix and recruitment of supporting cells like smooth muscle cells and pericytes by for instance platelet-derived growth factor (PDGF)-B and Tie-2. The process of angiogenesis is mainly associated with formation of microvascular networks.

1.2.2 Arteriogenesis

Arteriogenesis is the process of increasing the lumen of a pre-existing vessel to form collaterals[6]. This formation of collaterals includes recruitment and invasion of circulating

blood cells, proliferation of cells in the vessel wall and remodeling of the vessel in order to withstand larger blood flow. After occlusions of a supplying artery, arterioles become large conductance collateral vessels that maintain blood flow. Once an occlusion in a main artery takes place, blood flow is directed towards low resistance in the periphery via pre-existing arterioles. This redirection of blood flow increases shear stress at the arteriolar wall and activation of shear stress-responsive receptors on the EC membrane. Once activated, EC from the arteriole divert from a quiescent non-adhesive monolayer to a highly adhesive monolayer for circulating blood leukocytes by expressing different adhesion molecules and chemokines. With the help of selectins leukocytes can role over the endothelium and slow down and firm adhesion is achieved by interaction of integrins on the leukocytes and the adhesion molecules on the EC. Leukocytes can invade the vessel wall due to vascular permeability. The recruitment of monocytes has been extensively studied and is crucial for arteriogenesis. By using an ischemic hindlimb animal model in which new collaterals are formed due to a ligated femoral artery, several groups have shown that when monocytes are depleted from the animal the formation of collaterals is diminished and subsequently also the recovery of the blood flow[7,8]. This process seems to be dependent on the monocytes chemo attractant protein-1 (MCP-1) and the CCR-2 receptor signaling[9,10]. Stabile *et al*, showed that lymphocytes are mediators of collateral formation as diminished blood flow recovery in a hindlimb model of T cell deficient mice could be recovered with injection of functional T cells[11]. The exact mechanisms and triggers for the entry of leukocytes into the vessel wall at arteriogenesis still need to be further explored. These mechanisms together with EC sensing shear stress will drive a cascade of signaling transduction events that drive transcription factors and induce cellular responses. Important transcription factors induced by shear stress are activator protein-1 (also induced by inflammatory responses) and transcription factors of the Ets-family (driving VEGF and Tie expression). These transcription factors then drive events that induce EC and SMC replication. The vascular wall of the arteriole is remodeled by metalloproteinases that also help to create the space needed for vessel enlargement. Growing collateral arteries have a corkscrew-like pattern because of a relatively quick growth in length between two already fixed points. The collaterals are initially tortuous in order to compensate for the still increased shear forces, but the collateral eventually becomes indistinguishable from a normal artery with a medial layer and normal reactivity.

1.2.3 Vasculogenesis

Almost a decade ago the group of Isner discovered another mechanism that was involved in neovascularization[5]. They described for the first time that cells from the peripheral blood could be isolated which had the capacity to differentiate into cells with EC properties *in vitro*. Interestingly, these cells *in vitro* resembled angioblasts of blood islands during embryonal development as further explained in figure 2. Since these early observations, progenitors of EC (EPC) are being studied extensively for their angiogenic properties and potential therapeutic application in angiogenesis/neovascularization as well as re-endothelialization.

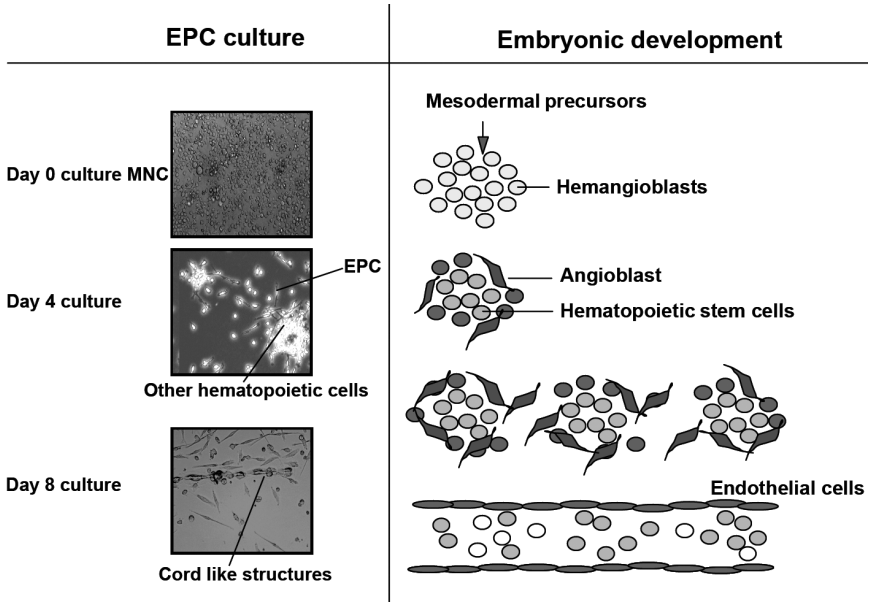

Figure 2: EPC cultures do resemble embryonic vasculogenesis.
Embryonic vasculogenesis begins as a cluster formation. The growth and fusion of multiple blood islands give rise to the yolk sac capillary network. After the onset of blood circulation, this network differentiates into an arteriovenous vascular system. The centers of these clusters will generate hematopoietic cells and are termed hematopoietic stem cells. Angioblasts are located at the periphery of the blood islands and are responsible for forming the vessel. EPC, derived from peripheral blood MNC fraction have properties similar to those of embryonic angioblast. At day 4 of culture, EPC can be defined as spindle shaped migratory endothelial cells. A great number of spindle shaped hemangioblasts can be seen. These hemangioblasts differentiate into EPC and hematopoietic stem cells (HSC). The HSC detach from the plate during culture and the attached EPC can be cultured further. After short term culture of 8 days cordlike structures of the spindle shaped EPC are formed.

1.3 Endothelial progenitor cells: Origins and differentiation.

The nature of the "true" circulating EPC is poorly defined. Different sources of EPC have been identified with each source having distinct properties[12,13]. In general, a common denominator for the different populations of cells that are termed EPC is the expression of EC-specific genes such as vascular endothelial-cell growth factor receptor-2 (VEGFR2).

1.3.1 Origins of EPC

Circulating EPC (CEP) that are characterized by the expression of the early hematopoietic stem cell markers CD34, CD133 and VEGFR2 can be recruited from the bone marrow[5,14,15]. CEP share these characteristics with hematopoietic stem cells and CEP have properties similar to the embryonic hemangioblast, which can give rise to both circulating blood cell lineages and vascular cells (Figure 2)[5,16]. Cultured with endothelial cell growth factors, purified CEP differentiate into endothelial-like cells that display a classic endothelial cell morphology and characteristics like the expression of von Willebrand factor (vWF), vascular endothelial (VE) cadherin and the capacity to take up acetylated low-density lipoprotein (acLDL). Although normally the number of CEP are limited (< 0,005% PB-MNC), their levels can be markedly elevated within days after the administration of CEP mobilizing agents[17], vascular trauma[18] or myocardial infarction[19,20].

Second, a subpopulation of peripheral blood mononuclear cells (PB-MNC) cultured on a gelatin- or fibronectin-coated dish in endothelial cell differentiation medium acquire the phenotype of endothelial cells within a short time period (4 to 7 days). These attaching cells, that are also referred to as EPC, display a spindle-like morphology and express endothelial cell markers like vWF, VEGFR2 and VE-cadherin. They are usually characterized by the binding of endothelial cell-specific lectins and the uptake of acLDL[12,13]. The large number of attaching cells that can be obtained from the PB-MNC cultures (up to 10%) make it unlikely that all these cells are derived from the low number of circulating $CD34^+$ cells. Most likely, these EPC are derived from more abundant subpopulations present in the mononuclear cell fraction like monocytes[13,21-24].

Several studies have shown that when these short term cultured EPC (4-7 days) were cultured further under EC growth conditions they could grow out into a monolayer of cells with cobblestone morphology resembling mature EC[25]. These so called late-outgrowth cells did exhibit true EC properties as they showed vWF positive staining in Weibel pallade

bodies and perinuclear anti-eNOS staining. They also showed a higher capacity to form tubular structures *in vitro* when compared to short term cultured EPC. Another source of EPC may be residing vascular progenitors so called "side population" cells (SP)[13,26]. Sainz *et al.* showed convincingly that vascular SP cells could under specific growth conditions differentiate towards EC-phenotype and they showed that SP cells formed complex vascular structures on a matrigel scaffold[13,26].

1.3.2 EPC differentiation

The exact differentiation cascade of EPC still needs to be elucidated[1]. It is currently unclear whether the different types of EPC are related through shared developmental stages, i.e. whether a monocyte-related intermediate is a required step in the differentiation of $CD34^+$ cells into EPC. The latter might be true as a recent publication showed that a subset of circulating monocytes have a low expression of CD34 antigen using a very sensitive new antigen detection method[27]. These $CD14^+CD34^{low}$ cells ranged from 0.6% to 8.5% of all peripheral-blood leukocytes and comprised most of the circulating KDR+ cells. Almost all $CD14^+$ BM cells were $CD14^+CD34^{low}$ double-positive cells with high multipotency and proliferation capacity when compared to the $CD34^-$ cells. It is clear that EPC are derived from hematopoietic stem cell (HSC) that reside in the bone marrow and can give rise to all blood types[28]. A model for EPC differentiation and how it fits in total hematopoiesis is depicted in figure 3. Important to notice is that EPC and EC do share the same myeloid lineage as Dendritic cells and Macrophages. This close relationship is further supported by the fact that these three terminally differentiated cells do share many phenotypical characteristics[29,30]. Differentiation of EPC *in vitro* and *in vivo* might share many common factors, as EPC *in vitro* do depend on the angiogenic growth factors present in the culture medium and also on the adherent surface. It is very likely that *in vivo* the same happens. Depending on the factors secreted and exposed by the injured sites, the adhered progenitors will become EC or another myeloid cell. Physical factors might be important in the differentiation of progenitors towards EC as well, as it was shown recently that shear stress could possibly induce EPC differentiation[31]. When EPC were *in vitro* subjected to shear stress their expression of KDR and other endothelial characteristics was markedly upregulated, inducing a cascade of differentiation and proliferation signals.

A lot of contradictions are found in literature with regard to the capacity of EPC to incorporate in newly formed vessels and thus if EPC are true progenitors of EC or if they are cells secreting angiogenic factors. In ischemic tissue the incorporation efficacy of true

EPC differs from 0% till 50%[32,33]. A likely explanation for this difference could be the severity of the injury (the more ischemia the more recruitment the more incorporation) or the different isolation and subsequent culture of progenitor cells under special conditions can change their properties[18,33]. In many studies a significant perivascular accumulation of BM-derived cells was observed in areas of collateral artery growth and capillary growth. These "pericytic" cells stained positive for some angiogenic growth factors and chemokines[13] and therefore it is highly likely that the capacity of EPC to promote neovascularization can also be accomplished by angiogenic paracrine effects[13,33].

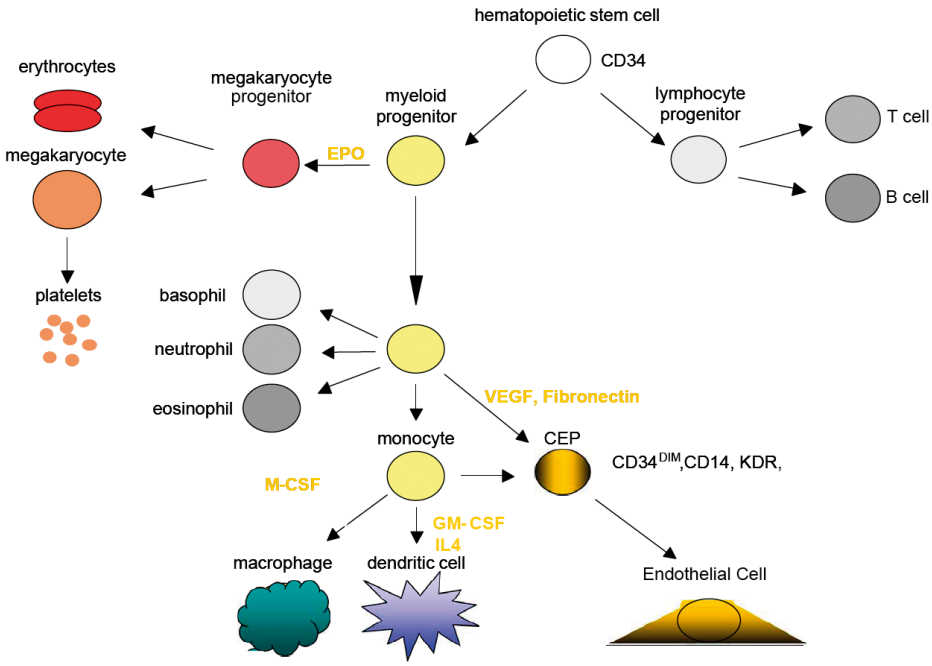

Figure 3: EPC in hematopoiesis.
EPC are thought to be derived from myeloid lineage and share common progenitors with the terminally differentiated DC and Mph.

1.4 Mobilization and homing of EPC

Figure 4 shows a schematic model for the concept of mobilization of EPC and their recruitment to sites of injury.

1.4.1 Mobilization of EPC

The mobilization of EPC from the bone marrow is regulated by various growth factors, cytokines and surface receptors. EPC are recruited from the bone marrow by factors that are secreted by ischemic or damaged tissue like VEGF and SDF-1[18,20,34,35]. Peripheral release of these growth factors activates MMP-9, that subsequently cuts membrane bound ckit resulting in the release of a soluble Kit Ligand[36]. $cKit^+$ progenitor cells can then move towards the vascular zone of the BM. Factors regulating proliferation and differentiation remain largely unclear but VEGF binding KDR might mediate further differentiation of the early KDR^+ progenitor. Endothelial specific nitric oxide synthase (eNOS) expressed by stromal cells in the BM has been shown to play an essential role in mobilization of EPC[37]. eNOS deficient mice show an impaired mobilization of progenitor cells, which was confirmed to be MMP-9 dependent.

1.4.2 Homing of EPC

Like other blood-leukocytes, EPC are attracted to sites of injury/ischemia by chemokines through their receptors. Locally delivered SDF-1 augments neovascularization *in vivo* by recruiting EPC to the ischemic site using their CXCR4 receptor[38]. The uptake of apoptotic bodies from mature EC could stimulate proliferation and differentiation of EPC, which could potentially be a important mechanism for differentiation at sites of injury[39]. That EPC home specifically to injured sites has nicely been shown by a tissue distribution study performed by Aicher et al, in which EPC were radioactively labelled and injected into athymic nude rats with an induced myocardial infarction showing that EPC homed predominantly to the infarct border zone[40].

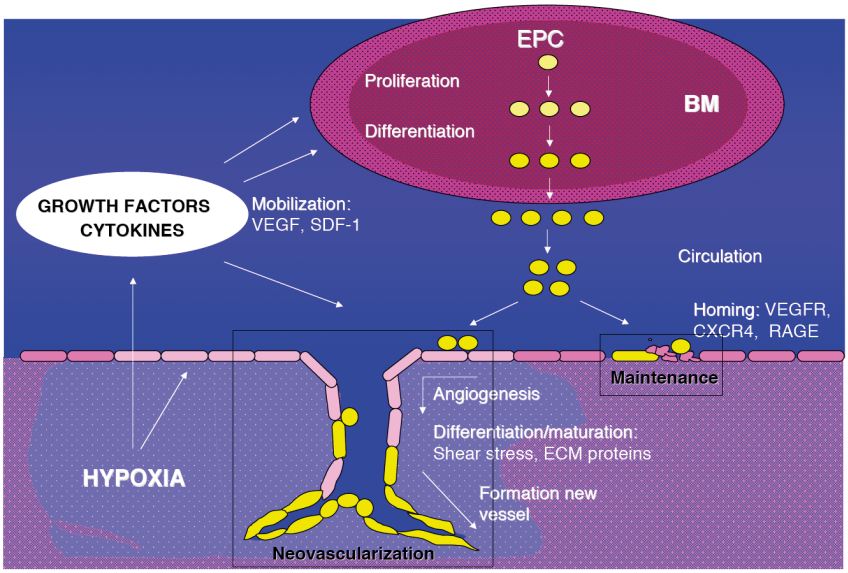

Figure 4: Schematic model of the concept of EPC mobilization and function.
HSC in the bone marrow are stimulated by angiogenic growth factors derived from damaged or ischemic tissue. They mobilize to the PB stream and cells can home towards chemokines depending on their receptors. Arrived at the site of injury they can either stimulate the already existing EC to proliferate (angiogenesis) or they can incorporate into the endothelium, stimulating vessel growth. Two therapeutic properties of EPC, neovascularization and regenerating the injured/damaged endothelium, are highlighted in separate boxes.

1.5 Therapeutic options of EPC: neovascularization and reendothelialization

The therapeutic capacity of EPC can be explored in two different directions. The angiogenic capacity of EPC can be used as therapeutic option to rescue critical ischemia in patients[41]. Or the capacity of EPC to differentiate to EC can be used for reendothelialization of damaged vessels and maintenance of the integrity of the endothelium[42,43].

1.5.1 EPC for neovascularization

The finding that human EPC incorporate in active sites of neovascularization[5] in animal models after induction of local ischemia has led to a number of transplantation studies using freshly isolated human $CD34^+$ (CB^{44} or BM recruited / $PB^{5,45-49}$), human $CD133^{+50,51}$,

murine SCA-1^{+} ^{48} cells and *ex vivo* cultured early[32,52-55] and late outgrowth EPC. This strategy of using local transplantation of (autologous) progenitor pools either directly obtained from bone marrow aspirates or from *ex vivo* cultured endothelial-like cells derived from the PB-MNC fraction has been explored widely preclinically as well as clinically. Preclinically, all cell-based studies confirmed a positive therapeutic efficacy of the angiogenic cells for neovascularization, however because these experiments were performed in different groups with different models it is hard to compare efficacies[13]. When early and late outgrowth cultured EPC were *in vivo* compared for their vasculogenic capacities they showed similar activities despite the higher angiogenic capacities of the late outgrowth EC *in vitro*. Furthermore, these preclinical studies suggested that EPC do have not-yet-defined but convincingly unique angiogenic characteristics, as terminally differentiated control cells (like human MVEC[32], Macrophages (Mph) and Dendritic cells (DC)[55] didn't show strong neovascularization capacities. In the last few years, there have been a couple of clinical transplantation trails using autologous cells for treatment of ischemic vascular disease using either BM-MNC[56-61], BM-CD133^{+} cells[62], G-CSF mobilized PB-MNC[63] or PB-MNC derived early outgrowth EPC with[56] or without G-CSF recovery[64]. There is some contradiction between these trials about the beneficial effects of the progenitor cells. Most of these trials show minor[57] beneficial effects while other trials show even adverse site effects[62,63] and this matter will be discussed further in chapter 8. There were some trials in which comparisons were made between transplantations with different pools of progenitor cells. For instance, when BM-MNC and PB-derived early outgrowth EPC were compared for their effect on remodeling of postmyocardial infarction. They both showed beneficial effects and to a similar extend[56]. Furthermore, in ischemic limbs, BM-MNC were found to be much better in recovering blood flow than PB derived MNC were[60].

1.5.2 Agents and methods to increase the number of EPC

Not all therapies have been focused on autologous transplantation of EPC. A lot of groups have focused on agents/methods that were capable of mobilizing EPC in order to increase the total circulating EPC number.

Two groups independently showed that physical exercise could increase EPC numbers and thus augment angiogenesis but also reduce neointima formation[65,66]. As already described, damaged tissue and hypoxia induced factors can mobilize EPC from the BM[18,20,34,35].

Administration of growth factors have been shown to increase the number of circulating EPC in experimental models as well as clinical pilot trials[67,68].

Intramuscular administration of VEGF using viral vectors enhanced levels of circulating EPC and restored the impaired neovascularization in ischemic hindlimbs of diabetic mice[69] . Similar to VEGF, basic fibroblast growth factor (bFGF), angiopoietin-1, SDF-1 and placental growth factor (PDGF) have also been shown to induce EPC mobilization and recruitment[70,71]. As EPC are thought to be myeloid cells derived from the $CD34^+$ hematopoietic stem cell, an additional method to increase circulating EPC is to use stem cell or myeloid cell recruiting factors such as granulocyte-colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage-colony-stimulating factor (M-CSF). Indeed, these growth factors have been shown to mobilize EPC and/or interfere in their differentiation[72,73]. However, as these cytokines have also an inflammatory key role and play part in atherosclerosis and restenosis, their safety has been questioned by recent studies[63,74]. Recently, a stimulatory effect of erythropoietin (Epo) has also been described on EPC recruitment and angiogenesis and as Epo has less inflammatory actions it might be a safer to use compared to other highly inflammatory cytokines[75,76]. In addition to the use of growth factors and cytokines to mobilize EPC from BM, several pharmacological agents have been proven to increase EPC levels. Statins induce mobilization and proliferation of circulating EPC *in vitro* and *in vivo* and they increase the amount of EPC incorporating at sites of neovascularization[77-79]. Statins were originally designed to reduce lipid levels in patients, however they have also proven very beneficial in reducing vascular inflammation[80]. Statins, but also many growth factors named above, have in common that they stimulate the Akt/PKB pathway and evidence is accumulating that the Akt/PKB pathway plays a central role in stem cell recruitment and survival of EPC.

Similar to statins, the glucose-lowering and anti-inflammatory peroxisome proliferator-activated receptor-γ agonists (PPARγ-agonists) can also enhance proliferation, differentiation and mobilization of EPC[81,82]. Interestingly, some BP-lowering drugs have also been shown to enhance angiogenesis in hypertension. For instance, ACE inhibitors were shown to increase capillary density, as much as VEGF, in an ischemic hind limb model[83] and enhanced EPC mobilization was also observed with ACE inhibition[84].

1.5.3 EPC for EC regeneration

It is very important to keep a good integrity and function of the endothelium, as injury and damage of the endothelium leads to inflammatory responses that can induce the formation of atherosclerotic lesions, plaque rupture infarctions and eventually end organ damage[85]. Early support for a role of bone marrow derived EPC in vascular repair in humans stems from the observation that the neointima formed on the surface of a left ventricular assist device accumulates a $CD133^+$ positive hematopoietic stem cell population that also expresses the endothelial cell marker VEGFR2[14]. In a mouse model, it was shown that bone marrow derived EPC can home to denuded arterial vessels and contribute to reendothelialization[86]. Likewise, infusion of BM derived $CD34^-/CD14^+$ did enhance and contribute significantly to endothelial regeneration[87]. The importance of these findings was further explored in animal models with high risk for atherosclerosis. For instance, hyperlipidemic Apolipoprotein $E^{-/-}$ mice show a lower number of endothelial progenitors in blood, which correlated with enhanced atherosclerosis[88].

The effect of transplantation of cultured early outgrowth EPC in reendothelialization was examined in rabbits, where a rapid reendothelialization of balloon-injured carotid arteries together with a reduced neointima formation was observed[89].

Augmenting the number of circulating EPC by recruiting them from the BM as discussed above is of course also beneficial for reendothelialization purposes and in that respect many EPC mobilizing agents have been studied as well in different animal models. Indeed, statin-induced mobilization of EPC was associated with an increased rate of reendothelialization and reduced neointimal thickening[86,90]. Likewise, PPARγ-agonist rosiglitazone promotes the differentiation of these EPC and attenuates neointimal formation in a mouse model with femoral angioplasty[82]. Mobilization of EPC with GM-CSF resulted in a reendothelialization and inhibited monocyte infiltration in an endothelium denuded artery in hypercholesterolemic rabbits[91].

It is not known if the beneficial effects of EPC are only due to reendothelialization or if EPC can facilitate other roles in atherosclerosis. For instance, here have been reports that EPC might contribute to facilitating plaque instability by inducing plaque angiogenesis[92].

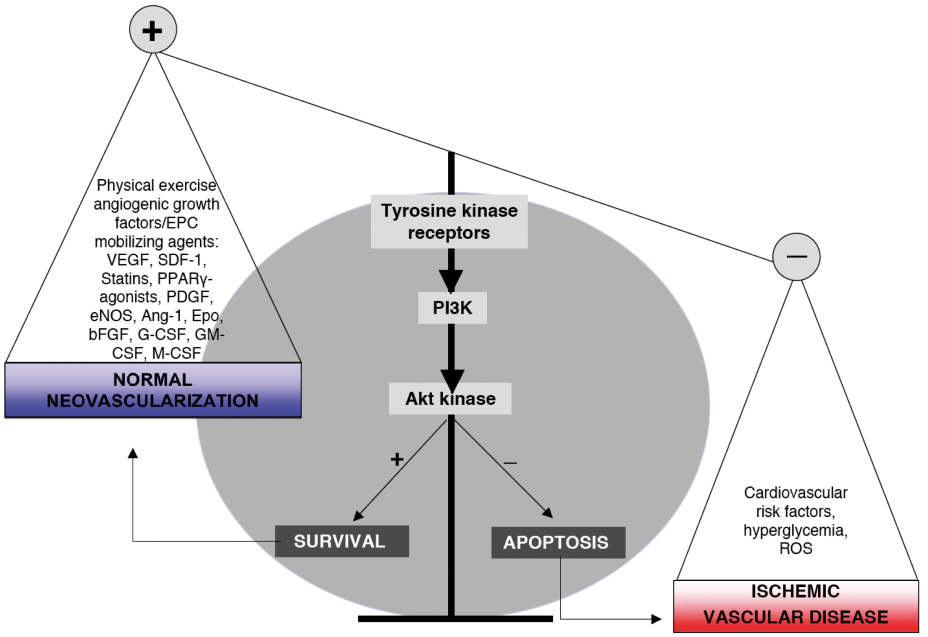

Figure 5: Keeping a tight balance of the number of EPC might prevent ischemic vascular disease.
As the number of EPC is diminished in patients suffering from classical risk factors, one could hypothesize that treatment with EPC mobilizing agents and angiogenic growth factors to keep the EPC number up could be beneficial. A key mechanism involved in the survival, proliferation apoptosis of the EPC is the PI3 kinase/ Akt pathway.

1.6 EPC dysfunction in ischemic vascular disease

1.6.1 EPC dysfunction in IVD

From the above it can be concluded that EPC of different hematopoietic lineages appear to play a crucial role in neovascularization of ischemic tissue and in the maintenance of endothelial cell integrity in injured vessels. Following these insights, it was hypothesized that impaired EPC function would predispose to endothelial cell dysfunction and its clinical manifestations including premature atherosclerosis and ischemic vascular disease. Seminal observations supporting this concept were reported by Vasa *et al*. who demonstrated that the number and function of circulating endothelial progenitor cells inversely correlated with risk factors for coronary artery disease[93]. It was shown that this concept holds true both for CD34 and VEGFR2 double positive CEP as well as for PB-MNC derived attaching EPC.

Hill *et al.* extended this observation showing that, for patients at risk for CVD, there was a strong inverse correlation between the number of EC colonies that could be obtained from PB-MNC cultures and the subjects' combined Framingham risk factor score[94]. Moreover, measurements of flow-mediated brachial-artery reactivity revealed a significant relation between endothelial function and the number of progenitor cells. In addition, EPC from the higher risk score patients revealed a more rapid senescence than those from the lower score, indicating a possible exhaustion of EPC that can contribute to the pathogenesis of the vascular disease. Furthermore, these reports again suggest that the quality of the endothelium may well be related to the endothelium-regenerative potential of circulating EPC.

1.6.2 EPC dysfunction and hyperglycemia

Schatteman and colleagues were the first to report data supporting the concept of EPC dysfunction in streptozotocin-induced diabetic nude mice[49]. Using an established model for neovascularization of the ischemic hindlimb they demonstrated that, as shown before in non obese diabetic mice, restoration of blood flow was significantly impaired in the diabetic mice. Whereas injection of human $CD34^+$ cells, purified from the PB-MNC fraction, did not accelerate the rate of neovascularization in healthy controls, it markedly enhanced blood-flow restoration in the diabetic mice. When labeled, $CD34^+$ cells were found to incorporate in the vasculature of previously ischemic tissue. It was concluded that in diabetic mice EPC function was deficient and that this could be corrected by transplantation of exogenous human $CD34^+$ cells. These data indirectly provided evidence for deficient EPC function in experimentally diabetic mice and initiated subsequent studies to investigate the nature of the EPC dysfunction.

Diabetes-associated metabolic factors may affect EPC function at several levels, including the number of available progenitor cells with capacity to differentiate into cells of the endothelial cell lineage, their capability to adhere and migrate to sites of reendothelialization and neovascularization and their pro-angiogenic (paracrine) potential.

1.7 Vascular problems in type-1 Diabetes Mellitus.

The metabolic disorder Diabetes Mellitus (DM) is characterized by chronic hyperglycemia due to reduced insulin-stimulated glucose uptake and/or impaired insulin secretion by beta cells in the pancreas. This thesis mainly focuses on the hyperglycemic state resulting from destruction of the insulin-producing β cells in humans (type 1 Diabetes Mellitus) and mice (streptozotocin-induced diabetes).

Hyperglycemia is one of the adverse metabolic risk factors associated with an increased risk of vascular disease. Type 1 diabetes is not only associated with microvascular complications[95] but also with premature atherosclerosis and a reduced capacity to form collateral vessels after an ischemic insult[96,97]. Likewise, patients with type 1 diabetes have an increased risk of clinical consequences of macrovascular disease including myocardial infarction and peripheral vascular disease[98]. Numerous studies have shown that dysfunction of the vascular endothelium plays a central role in the pathophysiology of diabetic microangiopathy and macroangiopathy[99]. The metabolic abnormalities that characterize diabetes, particularly hyperglycemia, provoke molecular mechanisms that have a major impact on endothelial cell function and survival. For instance, activation of protein kinase C (PKC) and increased oxidative stress can lead to endothelial cell dysfunction[100]. Moreover, prolonged exposure of endothelial cells to these adverse conditions increases endothelial cell apoptosis and turnover[101]. Although adjacent mature endothelial cells have the capacity to proliferate and replace these dying cells, chronic exposure to oxidative stress has been shown to lead to premature replicative senescence and limits this form of endothelial repair[102,103]. Eventually, endothelial cell death and shedding may lead to disturbances of the endothelial monolayer leaving a highly pro-atherogenic luminal surface[42,104].

1.8 Hypothesis and questions of the thesis.

Taken the vascular problems in patients with type 1 diabetes and the potential importance of EPC in maintenance and repair of injured endothelium and neovascularization of ischemic tissue into consideration, we hypothesized that EPC dysfunction reduces the vascular regenerative potential and thereby contributes to the pathogenesis of ischemic vascular disease. Thus, this thesis explores EPC dysfunction in diabetes mellitus focusing on angiogenic capacity, EPC differentiation and interventions to improve EPC function.

Questions addressed in this thesis are:

Chapter 2: How do different angiogenic cell subsets participate in neovascularization?

Chapter 3: Does EPC dysfunction exist in type-1 Diabetes Mellitus?

Chapter 4: Could elevated oxidative stress be involved in EPC dysfunction in Diabetes Mellitus?

Chapter 5: By using a transgenic mouse model, several questions could be answered.
Can EPC differentiation be tracked and how many cells are true EPC in short-term cultures?
How can EPC be distinguished from other myeloid differentiation lineages (macrophages and dendritic cells) as there are large phenotypic and functional overlaps?
Which sub-fraction of the BM contains the progenitors for EPC? Can myeloid growth factors such as M-CSF and GM-CSF stimulate the expansion of EPC?

Chapter 6: How does hyperglycemia affect EPC precursors in the bone marrow?

Chapter 7: How does short-term treatment of type-2 Diabetes patients with PPARγ-agonists affect the proinflammatory phenotype of monocytes and dysfunctional EPC?

Chapter 8: General discussion about the implications of the data presented in this thesis and suggestions for future studies.

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Chapter 2

CD34$^+$ Cells Home, Proliferate and Participate in Capillary Formation, and in Combination With CD34$^-$ cells Enhance Tube Formation in a 3-Dimensional Matrix

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Abstract

Objective

Emerging evidence suggests that human blood contains bone marrow (BM)-derived endothelial progenitor cells that contribute to postnatal neovascularization. Clinical trials demonstrated that administration of BM-cells can enhance neovascularization. Most studies, however, used crude cell populations. Identifying the role of different cell populations is important for developing improved cellular therapies.

Methods and Results

Effects of the hematopoietic stem cell-containing $CD34^+$ cell population on migration, proliferation, differentiation, stimulation of, and participation in capillary-like tubule formation were assessed in an *in vitro* 3-dimensional matrix model using human microvascular endothelial cells. During movement over the endothelial monolayer, $CD34^+$ cells remained stuck at sites of capillary tube formation and time- and dose-dependently formed cell clusters. Immunohistochemistry confirmed homing and proliferation of $CD34^+$ cells in and around capillary sprouts. $CD34^+$ cells were transduced with the LNGFR marker gene to allow tracing. LNGFR gene-transduced $CD34^+$ cells integrated in the tubular structures and stained positive for CD31 and UEA-1. $CD34^+$ cells alone stimulated neovascularization by 17%. Coculture with $CD34^-$ cells led to 68% enhancement of neovascularization, whereas $CD34^-$ cells displayed a variable response by themselves. Cell-cell contact between $CD34^+$ and $CD34^-$ cells facilitated endothelial differentiation of $CD34^+$ cells.

Conclusions

Our data suggest that administration of $CD34^+$-enriched cell populations may significantly improve neovascularization and point at an important supportive role for (endogenous or exogenous) $CD34^-$ cells.

Introduction

The formation of new capillaries plays a critical role in physiological and pathological processes such as wound healing, ischemia and tumor growth. It has long been thought that postnatal neovascularization occurred exclusively by migration and proliferation of preexisting endothelial cells (angiogenesis). Increasing evidence indicates that bone marrow (BM)-derived circulating endothelial progenitor cells (EPCs) are also involved in postnatal new vessel formation a process that[1], reminiscent of embryonic vessel formation, is termed adult vasculogenesis[2]. The concept of 'therapeutic vasculogenesis', administration of adult progenitor cells or progenitor-containing cell populations to stimulate neovascularization, and the potential of progenitor cells to serve as new vehicles for gene therapy has received a lot of scientific attention. Several small clinical trials aimed at therapeutic vasculogenesis by autologous transplantation of BM cells have been performed and improved clinical outcomes in patients with severe chronic limb ischemia or myocardial ischemia have been reported[3-6].

An important question concerning therapeutic vasculogenesis is, which cell population should be administered? Thus far, most clinical studies have used nonselected BM mononuclear cells; however, administration of such crude cell populations may have unwanted side effects. Recent data suggest that beside EPCs, BM contains other progenitor cells that may contribute to atherosclerosis[7], whereas hematopoietic cells were reported to have the capacity to produce profibrotic and angiogenic factors[8,9]. Better characterization of the BM cell subpopulation that can generate EPCs is of critical importance to development of safer and better-targeted cellular therapies.

Several studies have suggested that in human postnatal life, analogous to the existence of the embryonic hemangioblast[10], the $CD34^+$ hematopoietic stem cell population contains cells that can give rise to EPCs and endothelial cells[1,11,12]. This would suggest the potential use of $CD34^+$-enriched cell populations in therapeutic vasculogenesis. Various authors reported that administration of human leukocytes enriched for $CD34^+$ cells effectively enhanced neovascularization in animal models[1,13,14]. However, it has been argued that, because of lack of purity of the $CD34^+$-enriched cell populations and use of vital dye labeling of $CD34^+$ cells with possible dye transfer among cells, definitive proof that $CD34^+$ cells are angioblasts is lacking[15]. Several investigators pointed at a more important role for the $CD34^-CD14^+$ monocytic cell population[15-19]. Interestingly, recent reports suggest that

the majority of blood-derived cultured acetylated low-density lipoprotein (LDL) and *Ulex europeus* double-positive cells, which are commonly referred to as EPCs, may be derived from monocytes/macrophages[20]. We previously demonstrated that a minor but significant subset (≈9%) of cultured EPCs originates from the $CD34^+$ mononuclear cell population[21]. It has been suggested that EPCs derived from the monocyte/macrophage cell population exert their beneficial proangiogenic effects mainly by growth factor secretion and should be more appropriately called circulating angiogenic cells (CACs), whereas the hematopoietic stem cell-related populations may yield 'late outgrowth EPCs' in culture, also referred to as 'the true EPCs,' enhancing neovasculogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency[20-22].

The present study was conducted to determine the exact role of $CD34^+$ cells in human neovascularization. Thus far, most studies on human vasculogenesis were performed with human cells in small animal models lacking an intact immune system. We have used a 3-dimensional (3D) *in vitro* neovascularization model to study the role of human $CD34^+$ cells in vasculogenesis and angiogenesis in a human microvascular endothelial system under controlled circumstances. This model provides a tool to dissect the different mechanisms involved: migration, proliferation, differentiation, stimulation of, and participation in capillary formation.

Methods

Materials

Histopaque 1077, diaminobenzidine (DAB), KDR antibodies (Clone KDR1) and tetramethyl rhodamine isothiocyanate (TRITC)-labeled *Ulex europeus* were obtained from Sigma Chemicals, St. Louis, MO; DiI-labeled acLDL from Biomedical Technologies Inc, Stoughton, MA; histofine AEC solution from Nichirei, Tsukiji, Chuo-Kutokyo, Japan; VE-cadherin antibodies (clone BV6:MAB1989) from Chemicon, Temecula, CA; eNOS anitbodies from Transduction Laboratories, Lexington, KY; fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (clone8G12) antibodies, phycoerythrin (PE)-labeled goat anti-mouse IgG1-antibodies, FITC-labeled polyclonal goat anti-mouse antibodies and the Calibur flow cytometer from Becton Dickinson, Mountain View, CA; mouse anti-human CD31 antibodies, and anti-Ki67 antibodies (clone MIB-1) from DAKO, Glostrup,

Denmark; powervision goat anti-mouse poly-HRP-conjugates from Immunovision Technologies, Springdale, AR; FITC-conjugated streptavidin, TRITC-conjugated streptavidin, biotin blocking kit, ABC-mouse-kit, normal horse serum, biotinylated anti-mouse IgG, avidin DH solution, biotinylated peroxidase and vectashield from Vector Laboraties, Burlingame, CA; the magnetic cell sorter (MACS) from Miltenyi Biotec, Gladbach, Germany; StemPro-34-SFM medium (SCM), heat-inactivated newborn calf serum (NBCS) and fetal calf serum (FCS) from GibcoBRL, Grand Island, NY; stem cell factor (SCF), flt-3 ligand (FL), thrombopoietin (TPO) and basic fibroblast growth factor (bFGF) from Pepro Tech, Rocky Hill, NY; mouse anti-human LNGFR antibodies (clone 20.4) and the lymphoblast cell line Raji from ATCC, Manassas, VA; penicillin, streptomycin and medium 199 (M199) from Biowittaker, Verviers, Belgium; L-glutamine from ICN, Costa Mesa, CA; heparin from Leo Pharmaceutic Products, Weesp, The Netherlands; 0.4μm pore trans-well system from Costar, Cambridge, MA. Tumor necrosis factor-α (TNF-α) was a kind gift of Dr J. Travernier, Biogent, Gent, Belgium. Blocking antibodies against P-selectin (AF137), E-selectin (AF724), VCAM (BBA5) and ICAM-1 (BBA3) were all obtained from R&D Systems, Minneapolis MI.

Isolation of human CD34$^+$ cells

Mononuclear cells (MNCs) were isolated from human umbilical cord blood (hCB) by density centrifugation (Histopaque 1077). Cells were washed and CD34$^+$ cells were isolated by magnetic bead separation method (MACS). The residual cells, depleted from CD34$^+$ cells, served as CD34$^-$ cell population. The purity of the cell populations was analyzed by flow cytometry. Protocols for sampling hCB were approved by the institutional ethical committee. Written informed consent was obtained from all mothers before labor and delivery.

Genetic marking of CD34$^+$ cells

CD34$^+$ cells were cultured for 2 days with StemPro-34-SFM medium containing stem cell factor, flt3 ligand and thrombopoietin. Genetic marking was performed as described previously[23] using a retrovirus encoding the truncated low-affinity nerve growth factor receptor (LNGFR) as a marker gene. After transduction, CD34$^+$ cell selection was repeated as described to remove cells that had lost CD34 expression during transduction. Transduction efficiency and CD34$^+$ selection were evaluated by flow cytometry.

Flow cytometry

After MACS separation of $CD34^+$ cells the different cell populations were analysed by FACS. To evaluate the purity of the sorted cell populations, 25000 cells were stained FITC-conjugated anti-human CD34 and analyzed using a Calibur flow cytometer. As negative control the first antibody was substituted with an irrelevant murine IgG of the same subclass.

After transfection of $CD34^+$ cells with LNGFR, $CD34^+$ cell selection was repeated to remove cells that had lost CD34 expression during transduction. Aliquots of 25000 cells were incubated with mouse anti-human LNGFR-antibody. A PE-labeled goat anti-mouse IgG1-antibody was used as secondary antibody. Subsequently cells were incubated with FITC-conjugated anti-human CD34-antibody. Controls were included by substituting the primary antibody by an irrelevant antibody of the same subclass.

Differentiation and characterization of EPC/CAC

EPC/CAC were obtained as previously described[24] by culturing hCB-MNC at a density of $2*10^6$ cells/cm^2 on a gelatin-coated 6-well plate in M199 medium containing 20% fetal calf serum, penicillin/streptomycin, endothelial cell growth factor (ECGF), and heparin. At day 7, nonadherent cells were removed by thorough washing. Adherent cells were harvested and used in the 3D neovascularization assay as described.

To confirm endothelial phenotype, a subset of EPC/CAC were cultured on gelatin-coated coverslips for 7 days, fixed in cold methanol (-20ºC) and immunostained for the expression of the endothelial markers vascular endothelial (VE)-cadherin, endothelial nitric oxide synthase (eNOS) and kinase insert domain receptor (KDR) was performed using the ABC-mouse-kit. After blocking with normal horse serum for 30 minutes cells were incubated with the primary antibody (VE-cadherin, eNOS, KDR) at 37°C for 1 hour, followed by incubation with biotinylated anti-mouse IgG and normal horse serum for 1 hour at 37.5°C. Subsequently, the cells were incubated in 2% avidin DH solution and 2% biotinylated peroxidase for 1 hour at 37°C. As chromogen the histofine AEC solution was used. To assess the ability to take up acetylated LDL (acLDL) cells were incubated with DiI-labeled acLDL for 4 hours at 37°C and after fixation were incubated with FITC-labeled *Ulex europeus* agglutinin-I for 1 hour. For CD31-staining the fixed cells were incubated with mouse anti-human CD31 antibody for 1 hour, followed by incubation with FITC-labeled polyclonal goat anti-mouse antibodies. For all stainings controls were included in which the primary antibody was replaced by PBS or an irrelevant antibody of the same subclass.

In vitro neovascularization assay

Human foreskin MVEC were isolated, cultured, and characterized as described previously[25]. Cells were cultured until confluence at 5% CO_2/95% air on gelatin-coated dishes in M199 medium, supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum (serum pooled from 10 donors, obtained from a local blood bank), 10% heat-inactivated newborn calf serum (NBCS), 150 µg/mL endothelial cell growth factor (ECGF, prepared from bovine brain), 5 IU/mL heparin, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C. Subcultures were obtained by trypsin/EDTA treatment of confluent monolayers at a split ratio of 1:3. The experiments were performed with confluent cells ($0.7*10^5$ cells/cm^2) from passage 10-11 that were cultured without growth factor for at least 24 hours.

The *in vitro* neovascularization assays were performed in human fibrin matrices as described previously[26]. In short, highly confluent hMVECs ($0.7*10^5$ cells/cm^2) were seeded in a 1.25:1 split ratio on fibrin matrices and cultured in M199 medium supplemented with 10% human serum, 10% newborn calf serum, penicillin/streptomycin, basic fibroblast growth factor (bFGF) and tumor necrosis factor-α (TNF-α). After 24 hours, the medium was replaced with medium containing the mediators, with or without different cell populations. Fresh medium was added every second day. Invading cells and tubular structures of hMVECs in the 3D fibrin matrix were analyzed by phase-contrast microscopy. The length and amount of the tube-like structures was determined using an Olympus-CK2 microscope equipped with a monochrome charge-coupled device camera (MX5) connected to a computer with Optimas image analysis software. Six fixed microscopic fields (7.3 mm^2 per field) per well were analyzed and used to calculate the total length of the tube-like structures, expressed as mm/cm^2.

The effects of $CD34^+$ cells on in vitro capillary formation: homing to foci of neovascularization

To compare different cell populations for their migrational behavior on the angiogenic hMVEC monolayer, cells were added to the hMVEC culture medium in concentrations of 1% and 10% (expressed as a percentage of the total number of hMVECs seeded on the fibrin gel) 24 hours after seeding of the hMVEC. The following cell populations were studied: $CD34^+$ cell-enriched hCB-MNC (1%$CD34^+$, 10%$CD34^+$); $CD34^+$ cell-depleted hCB-MNC (1%$CD34^-$, 10%$CD34^-$); and human umbilical vein endothelial cells (HUVEC) and cells from a lymphoblast cell line (Raji) as proliferating nonangioblast leukocyte-like

cell population[27]. The hCB-MNCs were obtained from 5 donors. All experiments were performed in duplicate. The cultures were evaluated 7 days after addition of the cells to the hMVEC culture by 2 independent and blinded observers using phase-contrast microscopy. Additionally, immunohistochemical analysis of cross-sections was performed.

The movement of $CD34^+$ cells on MVEC monolayers was recorded by time-lapse video phase contrast microscopy during the initial 8 hour period (1 frame per 30 seconds). The movement of individual cells was followed in time. The number of $CD34^+$ cells that reached and those that remained stuck at tubular structures were counted. The number of cells that entered and remained in randomly taken comparable areas without capillary tubes were evaluated as controls.

Blocking antibodies against P-selectin (25µg/ml), E-selectin (50µg/ml), vascular cell adhesion molecule (30µg/ml), and intercellular adhesion molecule-1 (10µg/ml) were added 30 minutes before $CD34^+$ cells and their effects on $CD34^+$ cell accumulation at tubular structures were evaluated after 24 and 72 hours (medium was renewed after 24 hours).

Participation in new capillary formation

To investigate whether $CD34^+$ cells participate in the formation of new capillary-like tubes, LNGFR-transduced $CD34^+$ cells were used. LNGFR-transduced $CD34^+$ cells and nontransduced $CD34^+$ cells were compared for phenotypic and functional characteristics. LNGFR-transduced $CD34^+$ cells were added to the hMVEC monolayer alone and together with $CD34^-$ cells. After 7 days, cultures were terminated and prepared for immunohistochemical analysis.

Stimulation of new capillary formation

The effects on 3D capillary-like tube formation were studied for $CD34^+$ cells alone (1% $CD34^+$), $CD34^+$ cells together with $CD34^-$ cells (1% $CD34^+$/10% $CD34^-$, ie, ratio of 1:10, whereas the physiological ratio is 0.5:99.5), $CD34^-$ cells alone, EPC/CAC (1%), HUVECs, and Raji cells. The effects of subpopulations of hCB-MNC on new capillary formation were assessed in 9 independent experiments (9 hCB donors). All experiments were performed in duplicate. Cell populations were added to the hMVEC culture medium 24 hours after seeding of the hMVEC monolayer. Cultures were evaluated 7 days later. Total tube length was measured as described and expressed as percentage of tube length formed by bFGF/ TNF-α-stimulated hMVECs.

Immunohistochemical analysis

Fibrin matrices were fixed and embedded in paraffin. Sections (5µm) were stained for LNGFR to identify labeled $CD34^+$ cells and were analyzed by light microscopy. The number of $LNGFR^+$ cells containing a nucleus was counted and averaged over 5 cross-sections by a blinded observer and expressed as a percentage of the total amount of nucleus-containing cells in the monolayer.

To study proliferation of $LNGFR^+$ cells *in situ*, consecutive sections were stained for LNGFR or Ki67 (expressed on all proliferating cells during late G1, S, G2, and M phases of the cell cycle)[28]. Fibrin matrices were fixed at 4°C in 2% p-formaldehyde in PBS (pH=7.4), and embedded in paraffin. Serial sections were cut (5µm) perpendicular to the surface of the matrix sheet. Sections were deparaffinated, blocked in buffer containing 1.5% hydrogen peroxide and incubated in citrate buffer of 100°C for 20 minutes. Sections were washed and incubated with 150µl primary antibody (anti-LNGFR or anti-Ki67) for 60 minutes at 21°C. After washing sections were incubated with 150µl PowerVision goat anti-mouse poly-HRP-conjugates for 20 minutes 37°C. Diaminobenzidine was added as colouring substrate. Sections were counterstained with hematoxylin and mounted with pertex. Negative controls included substitution of the primary antibody with PBS or with an irrelevant murine IgG of the same subclass. Sections were analyzed by light microscopy. The number of $LNGFR^+$ cells containing a nucleus was counted and averaged over 5 cross-sections by a blinded observer. The amount of $LNGFR^+$ cells containing a nucleus was expressed as a percentage of the total amount of nucleus containing cells in the monolayer.

Immunofluorescence double-staining

Immunofluorescence double staining for LNGFR and CD31 or *Ulex europeus* lectin was performed to confirm the endothelial phenotype of $LNGFR^+$, $CD34^+$ cell-derived cells in newly formed capillaries. For LNGFR/CD31 double-staining a biotin-streptavidin detection system was applied. After blocking of endogenous biotin activity, sections were incubated with 100 µl of the first biotinylated anti-LNGFR primary antibody for 60 minutes, followed by incubation with 100 µl FITC-conjugated streptavidin for 30 minutes at room temperature. To augment the signal, sections were subsequently incubated with 100 µl anti-streptavidin-FITC for 30 minutes. Remaining biotin binding sites were blocked and sections were incubated with 100 µl biotin-labeled anti-CD31 for 60 minutes followed by incubation with 100 µl TRITC-conjugated streptavidin for 30 minutes at room temperature. Sections were air-dried and mounted with Vectashield. Controls were included in which the

procedure was performed with omission of the primary antibody or substitution of the primary antibody with an irrelevant murine IgG of the same subclass.
For LNGFR/*Ulex europeus* double staining, sections were incubated in 100 µl anti-LNGFR for 30 minutes, followed by incubation with 100 µl FITC-labelled polyclonal goat anti-mouse for 30 minutes at room temperature. Subsequently, the sections were incubated in 100 µl TRITC-labelled *Ulex europeus* for 30 minutes at room temperature and air-dried and mounted with Vectashield.

Interaction between CD34$^+$ and CD34$^-$ cells

To investigate the interaction between CD34$^+$ and CD34$^-$ cells, we performed several differentiation experiments. Differentiation cultures to obtain EPC/CAC were performed as described. CD34$^+$ cells were cultured in the presence or absence of CD34$^-$ cells (ratio 1:100). Subsequently, cultures were performed in which CD34$^+$ and CD34$^-$ cells were separated by a 0.4µm pore trans-well system allowing diffusion of soluble factors without physical contact between CD34$^+$ and CD34$^-$ cells. Differentiation was expressed as total number of attached cells per mm^2 or as number of attached cells relative to the number of cultured cells.

Statistical analysis

The data are expressed as mean ± standard error of mean (SEM) unless otherwise specified. Because of variations in basal tube formation between different batches of hMVECs, stimulation of capillary-like tube formation is expressed as percentage of tube length formed by bFGF/TNF-α-stimulated hMVECs (ie, versus 'control' culture). To determine whether the different cell populations (CD34$^+$, CD34$^-$, CD34$^{+/-}$ derived from the same 9 donors or EPCs, derived from 4 different donors) stimulated capillary-like tube formation significantly as compared with the reference value of 100%, 1-sample *t* tests with Bonferroni correction were performed. To compare the growth stimulating effects of CD34$^+$ and CD34$^{+/-}$ cells, which were all derived from the same 9 donors, a paired *t* test was performed. To compare the growth stimulating effect between CD34$^+$ or CD34$^{+/-}$ cells and EPC, which are derived from 4 different donors, separate independent sample *t* tests with Bonferroni correction were used. $P<0.05$ was considered statistically significant.

Results

Isolation and selection of CD34$^+$ cells

Flow cytometry demonstrated that in the cell population that was enriched for CD34$^+$ cells, 90.1±1.8% of cells were CD34$^+$. The CD34$^-$ cell population was largely depleted from CD34$^+$ cells (99.8±0.1% CD34$^-$ cells).

Characterization of EPC/CAC

Adherent EPC/CAC after 7 days of culture were characterized by immunohistochemistry. More than 90% of cells displayed a spindle-shaped morphology and stained positive for endothelial nitric oxide synthase, VE-cadherin, VEGFR2 (KDR), CD31, *Ulex europeus* lectin, and DiI-acetylated LDL uptake. All controls were negative. In long-term cultures (6 weeks), clusters and confluent monolayers of cobblestone-like cells were formed (Figure 1).

CD34$^+$ cells home to foci of neovascularization

hMVECs seeded on top of a fibrin matrix and cultured without addition of growth factors or cytokines remained as a quiescent monolayer on top of the matrix. When the hMVECs were exposed to both bFGF and TNF-α, they invaded the underlying fibrin matrix and formed capillary-like tubular structures (Figure 2A and 2C). When CD34$^+$ cell-enriched hCB-MNC were added to the hMVEC culture medium, formation of cell clusters was observed, specifically located at the sites of new capillary-like tube formation (Figure 2G and 2H). Addition of higher concentrations of CD34$^+$ cells resulted in larger cell clusters (Figure 2B versus 2D and 2E). In contrast, no such clusters were seen in the cultures to which CD34$^-$ cells or HUVECs were added (data not shown). In the cultures to which cells from the Raji lymphoblast cell line were added, there was a large number of cells and cell clusters present already at day 2 (Figure 2F). However, they did not colocalize with newly formed capillary-like structures but were randomly scattered throughout the culture. Time-lapse video image microscopy during the first 8-hour period revealed that the CD34$^+$ cells moved randomly over the endothelial monolayer and remained stuck at the vascular structures once they reached them (Table 1). No accumulation was seen in randomly chosen control fields of the monolayers. Viability of the homing cells was confirmed using a calcein uptake assay. Addition of antibodies against P- and E-selectin, vascular cell adhesion molecule, and intercellular adhesion molecule-1, either alone or simultaneously,

did not reduce the accumulation of $CD34^+$ cells at the vascular structures (tested with 3 $CD34^+$ populations from different donors) (Figure 3).

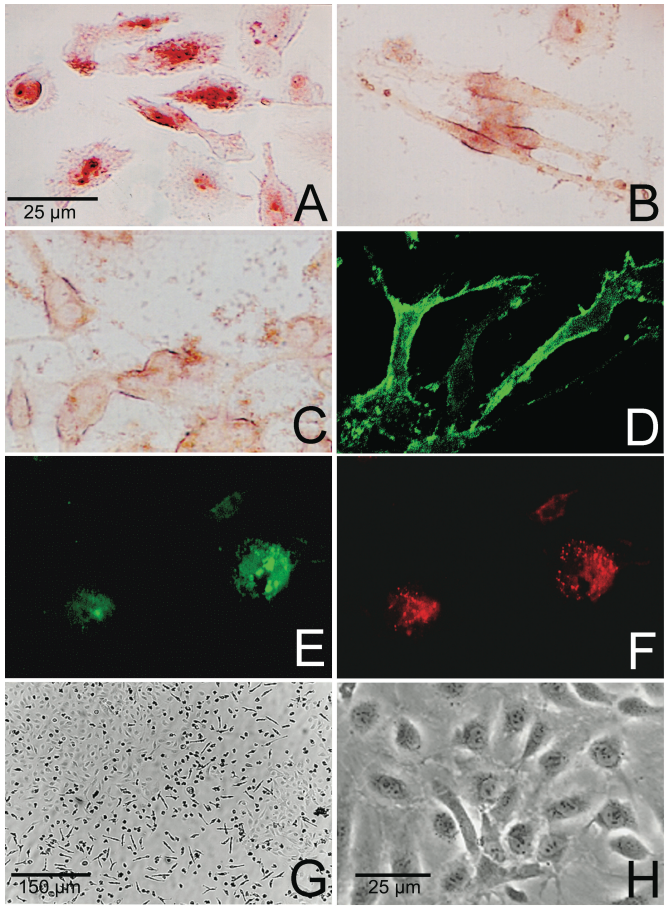

Figure 1

Characterization of EPC/CAC after 7 days of culture: expression of eNOS, perinuclear in the Golgi-system (A); VE-cadherin staining at cell-cell contacts (B); KDR staining (C); CD31 staining with typical membrane expression (D, confocal scanning laser microscopy). Ulex europaeus positive cells (E) take up DiI-labeled AcLDL (F) in double-staining experiments. In long-term cultures (6 weeks) formation of cell clusters and confluent monolayers of cobble stone-like cells is observed (G, H). Magnification (A-F, H) 630x; G 100x.

Table 1

	Experiment 1	Experiment 2	Cell Accumulation (% of Cells Entering the Area)
Tubular structures	13/16	27/34	80 ± 1%
Nonstructure monolayer (sum of 3 control areas):	3/56	5/130	4.6 ± 1.2 %

CD34+ cells accumulate specifically on endothelial tubular structures. Quantification of the accumulation of CD34+ cells was made by time-lapse video microscopy. CD34+ cells (104 cells per cm2) were added to monolayers of hMVECs that had been induced to generate tubular structures by incubation with bFGF and TNF-α. The movement of individual CD34+ cells over the endothelial monolayer was monitored between 2 and 8 hours after addition of the cells. To that end, 5 tubular structures in a field were encircled and the number of CD34+ cells that entered and left the marked areas were monitored. As controls, the surface areas of the 5 structures were projected in 3 directions to only monolayer areas without tubules or sprouting in the same microscopic field. The cell numbers of the 3 sets of control data were highly comparable and their sum is given for both experiments. Cell accumulation is given as the mean ± range.

CD34$^+$ cells participate in new capillary formation

To study the contribution of CD34$^+$ cells to new capillary-like tube formation, CD34$^+$ cells were retrovirally transduced with the marker gene LNGFR. The CD34$^+$ cell population that was genetically marked with the LNGFR gene contained 92±2.6% CD34$^+$ cells, which is similar to the nontransduced CD34$^+$ cell-enriched mononuclear fraction. Transduction efficiency with the LNGFR gene was 39.3±2.9%. After retroviral transduction with the LNGFR gene CD34$^+$ cells maintained their phenotypic and functional characteristics. Addition of transduced CD34$^+$ cells to hMVEC monolayers caused similar homing of cells to angiogenic sites as observed after addition of nontransduced CD34$^+$ cells. Addition of transduced CD34$^+$ cells to hMVEC monolayers also caused a stimulatory effect on bFGF/ TNF-α-induced new capillary-like tube formation that was similar to the effect observed after addition of nontransduced CD34$^+$ cells (data not shown). Immunohistochemical staining of cross-sections confirmed homing of LNGFR$^+$ cells in and around new capillary sprouts and showed that LNGFR gene-marked cells participated in new capillary-like structures (Figure 4A). The transduced cells were fully integrated in the angiogenic endothelium and stained positive for the endothelial markers CD31 and *Ulex europeus* lectin (Figure 4B to 4G). In several sections LNGFR$^+$ cells were observed deep in the tubular structures, suggesting a pioneering role of CD34$^+$ cells (Figure 4A and 4F). Double-staining with proliferation marker Ki67 showed that 25% to 40% of the LNGFR$^+$ cells were proliferating (Figure 4H and 4I). All controls were negative.

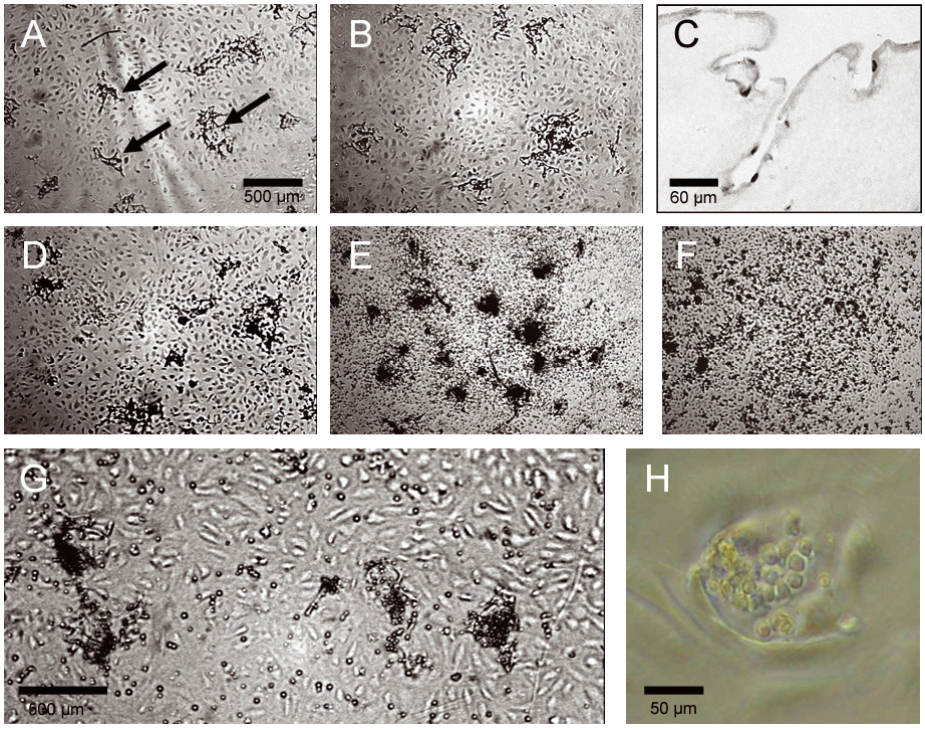

Figure 2

HMVECs were seeded at confluent density on top of a 3D fibrin matrix and stimulated with bFGF (5 ng/ml) and TNF-α (4 ng/ml). A,B,D,E Nonphase contrast photomicrographs after 7-day culture without (A) or with 1% CD34+ cells (B) or 10% CD34+ cells (D,E), which were added 24 hours after seeding the hMVECs. A, The arrows indicate sites of capillary-like tube formation by hMVEC. C, Cross-section of tubular structures at higher magnification. B,D,E, When 1% (B) or 10% CD34+ cells (D,E) were added to the hMVEC monolayer, clusters of CD34+ cells accumulated at sites of capillary-like tube formation. G, Cluster formation at angiogenic sites was visible after 4 days of culture after addition of 10% CD34+ cells (2x larger magnification). H, Early accumulated CD34+ cells at higher magnification. Addition of 1% or 10% CD34- cells or 1% or 10% HUVECs did not cause such cell clustering. F, Addition of Raji cells (10%) led rapidly to increased amounts of cells and cell clusters, which were randomly scattered throughout the culture without preference for tubular structures. Nonphase photomicrographs were taken after 7 days of culture except for (G) and (H) (phase contrast), which were taken at day 4 and 3, and (F), which was taken at day 2 because of the extensive cell proliferation and overgrowth. A-E, Data are representative photographs of 6 independent experiments using CD34+ cells from different donors. Similar results were obtained when tubular structures were induced by addition of VEGF (20 ng/mL) and TNF-α (4 ng/mL). A,B,D-F, Same magnification.

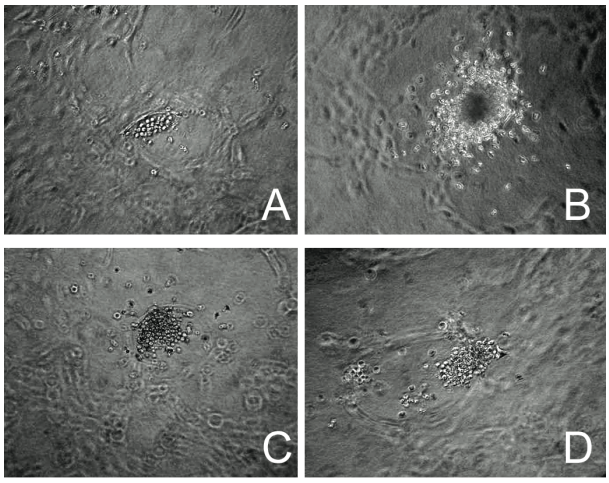

Figure 3

Cluster formation of CD34+ cells at sites of capillary-like tube formation. Culturing of cells in control medium (A); medium containing antibodies against P-selectin and E-selectin (B); medium containing antibodies against I-CAM and V-CAM (C); or medium containing antibodies against both P-selectin and E-selectin, as well as I-CAM and V-CAM (D), did not reduce CD34+-cell accumulation at vascular structures.

Quantification of LNGFR$^+$ cell participation in capillary formation suggested that addition of CD34$^+$ cells (1%) to the hMVEC monolayer led to a contribution of CD34$^+$ cells of 1.0 ±0.5% to the new vascular structures. This implies a significant incorporation of CD34$^+$ cells in the endothelial lining, which was estimated >10% of the added CD34$^+$ cells. The exact percentage of participation is difficult to estimate because CD34$^+$ cells proliferated during the 7 days of incubation.

It should be noted that the transduction efficiency of almost 40% causes a 2.5-fold underestimation of the total amount of participating CD34$^+$ cells.

CD34$^+$ cells stimulate neovascularization, which is further enhanced by coculture with CD34$^-$ cells

Figure 5 shows the effect of different hCB-MNC subpopulations on capillary formation in the 3D fibrin matrix. Addition of 1% EPC/CAC to the hMVEC-monolayer caused a 60 ±12% increase in capillary formation ($p<0.05$ versus controls). Overall, if CD34$^+$ cells alone (>90% purity) were added to the hMVEC monolayer, only a slight increase in new capillary-like tube formation was observed (17±5% increase compared with controls, $p<0.05$). When CD34$^+$ cells were added in combination with CD34$^-$ cells, a considerable increase of total tube length was found, which was similar to the effect of cultured EPC/CAC (68±15%; $p<0.05$). Overall, CD34$^-$ cells alone did not cause a significant increase in tube length (Figure 5A). However, 2 types of responses could be distinguished in the responding cells. In preparations of 6 different donors CD34$^-$ cells alone did not

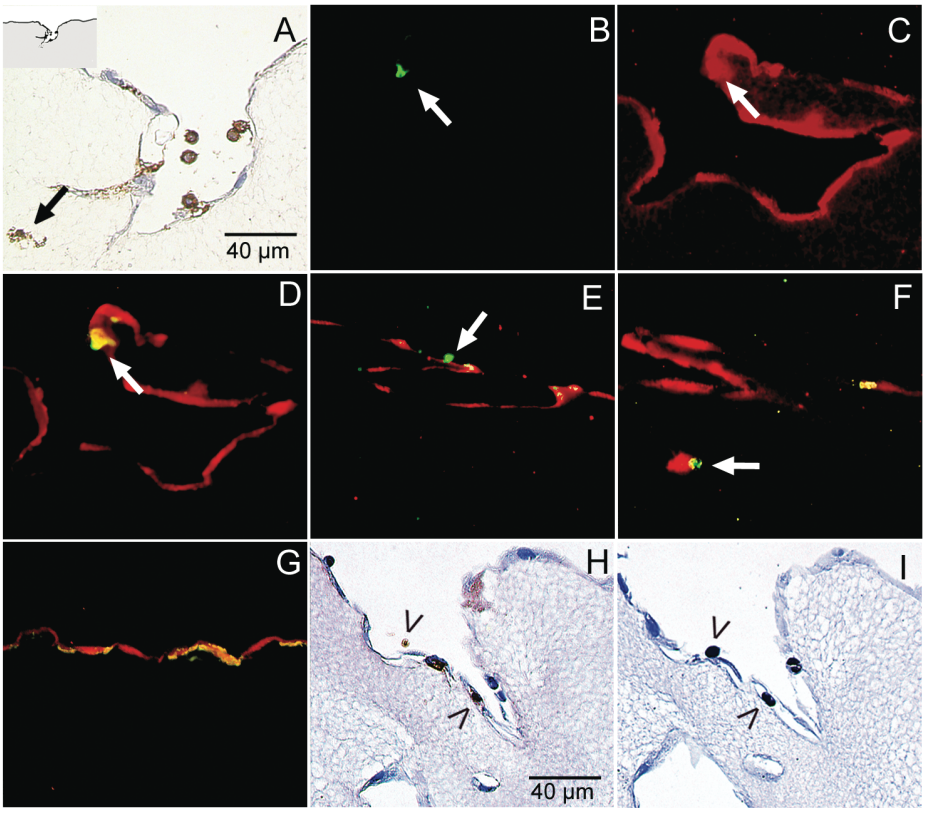

Figure 4

Histological sections showing homing, participation and differentiation of LNGFR gene-marked CD34+ cells in the capillary-like tubular structures in a fibrin matrix (400x). LNGFR gene-marked cells (LNGFR+ stained brown) home to sites of capillary formation, incorporate, become fully integrated in the endothelial cell monolayer, and acquire a phenotype that is morphologically similar to endothelial cells (A). LNGFR gene-marked cells can also be found deeper in the tubular structures in direct contact with the matrix (A, arrow). The small window within (A) gives a schematic overview. Immunofluorescent double-staining for LNGFR (green) (B) and Ulex europeus lectin (red) (C) strongly suggests that incorporated CD34+ cells differentiate into endothelial cells (yellow) (D). Homing cells before incorporation do not stain positive for the endothelial cell marker (arrow) (E). Some of the cells at the tip of the vascular in-growth are negative for the endothelial cell marker, suggesting a higher angiogenic capacity of EPC in an earlier phase (F). Immunofluorescent double-staining for LNGFR (green) and CD31 (red) also implies endothelial differentiation of LNGFR gene-marked CD34+ cells (yellow) (G). Histological analysis of consecutive cross-sections of capillary-like tubular structures shows the presence of proliferating LNGFR gene-marked cells. H, LNGFR staining (brown). I, Corresponding consecutive cross-section, stained for Ki-67 nuclear antigen, expressed on proliferating cells (dark nuclear staining). The open arrows indicate LNGFR-Ki67 double-positive cells, indicating the presence of proliferating CD34+ cell-derived cells.

significantly increase tube length. In these preparations, stimulation of growth of vascular structures was only observed when $CD34^+$ and $CD34^-$ cells were added together, whereas none of the separate preparations induced a significant response (Figure 5B). In the preparations of 3 other donors the $CD34^-$ cells were able to increase the extent of endothelial tube formation by themselves, which could not be further enhanced by the presence of $CD34^+$ cells (Figure 5C). The latter finding suggests that in certain circumstances, for example when $CD34^-$ cells are in an activated state, $CD34^-$ cells may have a predominant effect on the observed tube length enhancement. Neither HUVEC nor cells from the Raji cell line significantly influenced total tube length.

Interaction between $CD34^+$ and $CD34^-$ cells

When isolated $CD34^+$ cells were cultured alone, no differentiation into EPC occurred (0 ±0%). However, coculturing of $CD34^+$ cells with $CD34^-$ cells resulted in a significant increase in EPC differentiation (122±11/mm^2, $p<0.000$ versus $CD34^+$ cells alone). To assess whether this increase in differentiation rate was induced by soluble growth factors, $CD34^+$ cells were cultured separated from $CD34^-$ cells by a membrane that allowed soluble molecules to diffuse freely but prohibited physical contact between $CD34^+$ and $CD34^-$ cells. EPC differentiation from $CD34^+$ cells in these cultures was rarely observed (6±1/ mm^2, $p=1.0$ versus $CD34^+$ cells alone). Subsequently, experiments were performed in which $CD34^+$ cells were labelled with PKH-26. Previous experiments showed that with this labelling method under these specific culturing conditions for a period of 7 days minimal dye transfer (<0.1%) occurred. We observed that coculturing of $CD34^+$ and $CD34^-$ cells led to a significant increase in differentiation of $CD34^+$ cells to EPC as compared with cultures in which $CD34^+$ cells were cultured alone or cultures in which $CD34^+$ and $CD34^-$ cells were separated using a transwell system (72±9% versus 0% or 1.5±0.3%; $p<0.05$). These experiments suggest that cell-cell contact between $CD34^+$ and $CD34^-$ cells is necessary for endothelial differentiation of $CD34^+$ cells.

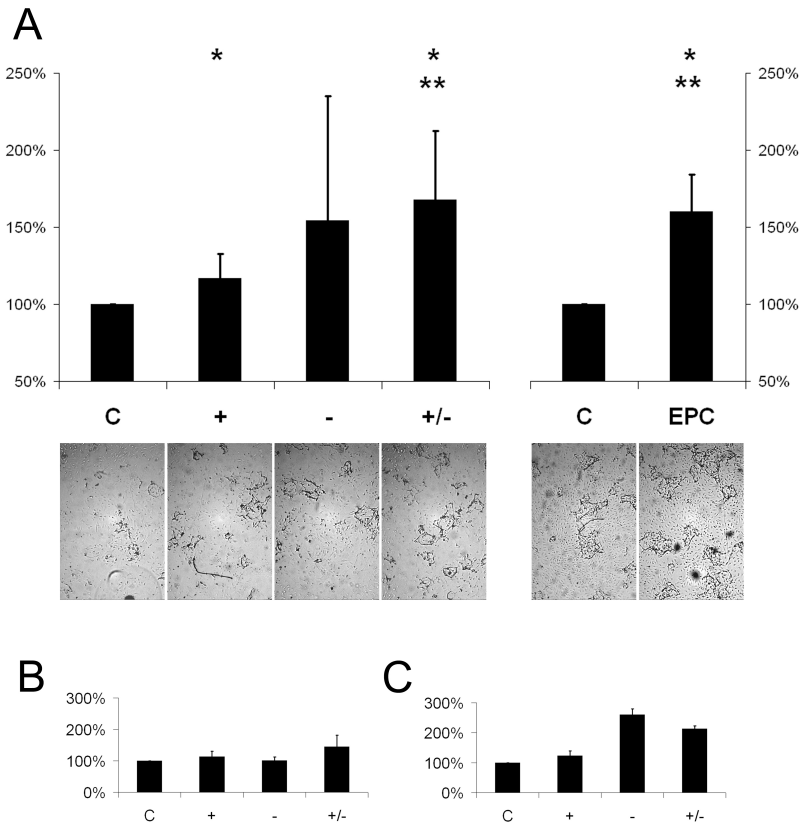

Figure 5

Effects of different subpopulations of hCB-MNC on capillary-like tube formation. The left part of (A) shows the effects of different subpopulations of hCB-MNC on capillary-like tube formation expressed as a percentage of tube length formed by bFGF/TNF-α-stimulated hMVEC. The nonphase photomicrographs show representative examples of the observed tube formation in the hMVEC monolayer in the presence of the respective hCB-MNC subpopulations. CD34+ cells were added to the hMVEC culture medium in a concentration of 1% either alone (+) or in combination with 10% CD34- cells (+/-) and compared with control cultures (bFGF/TNF-α-stimulated hMVEC without addition of cell-populations). Data are expressed as mean±SEM of 9 independent experiments (9 hCB donors). The right part of (A) shows the effects of hCB-derived cultured EPCs/CACs (EPC) on tube formation under the same conditions as the left panel and represent the mean±SEM of 4 independent experiments. NS indicates nonsignificant. * Significant increase compared with controls ($p<0.05$). ** Significant increase compared with 1%CD34+ stimulation ($p<0.05$). B and C, Effects of the 2 subpopulations of the hCB-MNC given in (A) on capillary-like tube formation, namely those in which there was no significant influence on tube formation by the sole addition of the CD34- cells (B; n=6) and the subset of the 3 CD34- cell preparations that were able to increase the extent of endothelial tube formation themselves.

Discussion

In the present study, we used a recently developed *in vitro* 3D model to study different aspects of human adult vasculogenesis under controlled circumstances and demonstrate that human $CD34^+$ cells selectively home to sites of angiogenic microvascular human endothelium, proliferate, become fully integrated in the microvascular endothelial monolayer, are able to differentiate into endothelial cells *in situ,* and have a mild but significant stimulatory effect on new capillary formation. Interestingly, if $CD34^+$ cells are cultured on an hMVEC monolayer in the presence of $CD34^-$ cells this results in marked enhancement of neovascularization similar to the effect of cultured EPC/CAC.

Although evidence is accumulating that vasculogenesis occurs in human postnatal life[1,12,29-31], the origin of the EPC is still under dispute. Previous studies suggested $CD34^+$ cells as main source of EPC but were criticized because of $CD34^+$-enriched cell populations of low purity and the possibility of vital dye transfer among cells. Our experiments, using $CD34^+$ cells of high purity (>90%) and a very stable cell labeling method, ie, retroviral transduction with the marker gene LNGFR which results in permanent insertion of the LNGFR gene in the cells, strongly support a role for $CD34^+$ cells as a source of EPC. Moreover, our data indicate that retroviral vectors can effectively transduce $CD34^+$ cells and that transduction with a marker gene does not influence their phenotype, homing, or angiogenic capacity. Together with the ability of purified $CD34^+$ cells to selectively home to angiogenic endothelium and incorporate in the endothelial monolayer, this suggests that $CD34^+$ cells hold promise for gene therapy applications.

Although in our study administration of a purified population of $CD34^+$ cells caused relatively modest integration of these cells in the hMVEC monolayer and only a mild stimulatory effect on neovascularization, it strongly suggests homing and incorporation of human $CD34^+$ cells in human microvascular endothelium. Thus far, integration of BM cells into the endothelium has been mainly assessed in animal models. Highly variable incorporation rates have been reported, probably because of differences between models in intensity of endothelial injury. Generally, the number of incorporated endothelial cells is quite low[32]. Thus far, a few animal studies have assessed the effects of injection human $CD34^+$ cells on neovascularization and incorporation in animal endothelium. Asahara et al were the first to report integration of DiI-labeled $CD34^+$ cells in 13% or 10% of capillaries in mouse or rabbit hindlimb ischemia[1]. Kocher reported incorporation of human $CD34^+$ cells in 20 to 25% of total myocardial capillary vasculature in a mouse myocardial

infarction model and considerable stimulation of neovascularization resulting in improvement of cardiac function[13]. In contrast, Schatteman et al demonstrated that human $CD34^+$ cells incorporated and differentiated into endothelial cells (not quantified) but did not influence restoration of blood flow in nondiabetic mice with hindlimb ischemia[14]. In the latter experiments administration of $CD34^+$ cells did cause a marked acceleration of flow restoration in diabetic nude mice, suggesting that the effects of injected $CD34^+$ cells on neovascularization may depend on (dys)function of the resident population of endothelial cells and/or angioblasts. In our model, we used a normal hMVEC population, which may already have had an optimal capillary-forming capacity. We cannot exclude the possibility that in the presence of resident endothelial cell dysfunction administration of $CD34^+$ cells alone may be more effective. However, this cannot fully explain the modest stimulatory effect of $CD34^+$ cells on neovascularization, as cultured EPC/CAC, administered in similar concentrations as $CD34^+$ cells, did markedly enhance neovascularization in our model.

An alternative explanation for the limited effect of purified $CD34^+$ cells in our *in vitro* model as compared with animal models might be the absence of (endogenous) supporting cells. Emerging evidence suggests that subsets of hematopoietic cells support capillary growth. Hematopoietic cells have been shown to release angiogenic factors such as VEGF, angiopoietins and MMPs. Additionally, subsets of recruited hematopoietic cells, particularly myelomonocytic cells, were shown to support EPC-mediated neovascularization[33]. Recently, several *in vitro* studies have suggested that loco regional interactions between $CD34^+$ and $CD34^-$ cells may be important for proliferation and differentiation of $CD34^+$ cells into endothelial cells[1,24,34]. Interestingly, in our model homing or incorporation of $CD34^+$ cells into the endothelial layer did not depend on the presence of $CD34^-$ cells, suggesting that angiogenic endothelium may not only express or secrete factors to recruit $CD34^+$ cells but also for their incorporation and proliferation. However, our data show that although separate administration of selected $CD34^+$ or $CD34^-$ cells for the majority of the donors had only limited effects on neovascularization, coincubation of both cell populations resulted in a consistent and marked increase (68%) in neovascularization, indicating that interactions between $CD34^+$ cells and $CD34^-$ cells can contribute to stimulation of capillary growth. Our observations that coculturing of $CD34^+$ cells with $CD34^-$ cells significantly enhances EPC differentiation *in vitro*, suggest that this is at least partly caused by enhanced differentiation and incorporation of $CD34^+$ cells.

Apart from ($CD34^-$ cell-stimulated) $CD34^+$ cell incorporation enhanced capillary growth after $CD34^{+/-}$ cell coincubation may be explained by a stimulatory effect of $CD34^+$ cells on

endothelial differentiation of $CD34^-$ cells. Several studies have demonstrated that $CD34^-$ cells may differentiate into endothelial cells *in vitro*[19,20,35]. $CD34^-$ cells may even be the main source of cultured EPCs[21]. Several studies reported only rare incorporation of $CD34^-$ cells into new capillaries *in vivo*[1,14] without functional improvements in blood flow. It has been suggested that $CD34^-$ and $CD14^+$ cells yield the 'early EPC,' which have no significant proliferative capacity and contribute to neovascularization mainly by secreting angiogenic cytokines[20,22]. However, Harraz et al[15] demonstrated that $CD34^-$ cells and $CD34^-/CD14^+$ cells may incorporate into the neovasculature *in vivo* when coadministered with $CD34^+$ cells. Such potential integration of $CD34^-$ cells is intriguing and warrants further study. However, our study was not designed to demonstrate or exclude endothelial differentiation or incorporation of $CD34^-$ cells. The aim of our present study was to investigate specifically the role of human $CD34^+$ cells in human neovascularization. We chose to use retroviral transduction with a marker gene in our $CD34^+$ cells because this is a very stable and reliable labeling method and because retrovirally transduced autologous $CD34^+$ cells have already been applied for clinical use. This labeling method is, however, not suitable for labeling of the $CD34^-$ cell population.

It should be noted that our study is confined to the formation of human capillary-like tubular structures *in vitro*. However, if confirmed, our findings have important clinical and therapeutic consequences. They suggest that administration of a $CD34^+$-enriched cell population may lead to a significant improvement of neovascularization, which is similar to the effects observed with cultured EPC/CAC. The use of $CD34^+$-enriched cells has the advantage that fresh administration of these cells after brief manipulation is much easier than culturing EPCs, which involves prolonged sterile manipulation. Furthermore, if cultured EPCs/CACs are indeed monocytes/macrophages, potentially harmful effects caused by their proinflammatory characteristics have to be taken into account. Finally, much experience has been obtained over the years as $CD34^+$ cell-enriched cell populations have been used extensively for treatment of hematological and nonhematological malignancies and *ex vivo* expansion methods have been developed[23,36,37]. Our observation that human $CD34^+$ cells selectively home and incorporate in angiogenic endothelium suggests their potential for gene therapy. At last, our observations suggest an important supportive role for the $CD34^-$ cell population. Further studies should be aimed at unraveling the interactions between $CD34^+$ and $CD34^-$ cells.

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Chapter 3

Endothelial Progenitor Cell Dysfunction: a Novel Concept in the Pathogenesis of Vascular Complications of Type 1 Diabetes

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Abstract

Type 1 diabetes is associated with reduced vascular repair, as indicated by impaired wound healing and reduced collateral formation in ischemia. Recently, endothelial progenitor cells (EPC) have been identified as important regulators of these processes. We therefore explored the concept that EPC are dysfunctional in Diabetes Mellitus. The number of EPC obtained from type 1 Diabetes patients in culture was 44% lower compared to age- and gender- matched controls ($P<0.001$). This reduction was inversely related to levels of HbA1c ($R=-0.68$, $P=0.01$). In addition, we demonstrated that patient EPC were also impaired in function using an *in vitro* angiogenesis assay. Conditioned media from patient EPC were significantly reduced in their capacity to support endothelial tube formation in comparison to control EPC. Therefore, despite culturing the EPC under normoglycemic conditions, functional differences between patient and control EPC were maintained. Our findings demonstrate that adverse metabolic stress factors in type 1 diabetes are associated with reduced EPC numbers and angiogenicity. We hypothesize that EPC dysfunction contributes to the pathogenesis of vascular complications in type 1 diabetes.

Introduction

Hyperglycemia is associated with endothelial cell dysfunction and reduced neovascularization in response to tissue ischemia, processes that are essential for wound healing and prevention of cardiovascular ischemia[1-3]. A growing body of evidence indicates that neovascularization does not exclusively rely on proliferation of local endothelial cells but also involves bone marrow-derived circulating stem cells[4]. These cells can be cultured from the circulating mononuclear cell fraction and are commonly referred to as endothelial progenitor cells (EPC) because they exhibit characteristic endothelial surface markers and properties. Moreover, a number of studies have shown that injected EPC home to sites of ischemia, incorporate into the newly formed capillaries and augment neovascularization[5]. Consequently, if EPC are critical to endothelial maintenance and repair, EPC-dysfunction could contribute to the pathogenesis of ischemic vascular disease. Indeed, studies have demonstrated that, in patients with cardiovascular risk factors, the number of EPC that can be isolated from peripheral blood is reduced[6] and EPC function is impaired[7,8]. It was recently reported that a strong inverse correlation exists between the number of EPC and the subjects' combined Framingham risk factor score[9]. In addition, measurements of flow-mediated brachial-artery reactivity also revealed a significant relation between endothelial function and the number of EPC, supporting a role for EPC in the maintenance of endothelial integrity.

In this study, we investigated the hypothesis that EPC dysfunction exists in type 1 diabetes patients. To that end, we determined the number of EPC obtained from peripheral blood of type 1 diabetes patients and its relation to glycemic control. Furthermore, we compared the capacity of patient and control EPC to support endothelial tube formation *in vitro*.

Research design and methods

Patient characteristics

After informed consent was obtained, peripheral blood (PB) samples were collected from twenty type I diabetes patients and twenty age- and gender-matched healthy control subjects. Patients with type 1 diabetes, diagnosed at least 1 year before entering the study,

were recruited from the Department of Diabetology of the University Medical Center Utrecht. All patients were treated with insulin for at least one year. Patients with manifest macrovascular disease were excluded. Other exclusion criteria included: smoking, alcohol abuse, liver disease, creatinine >120 μmol/l and untreated thyroid disease. If patients were treated with vasoactive medication (angiotensin-converting enzyme inhibitors, statins, aspirin, non-steroidal anti-inflammatory drugs, angiotensin II antagonists, folic acid or vitamins), treatment was stopped at least 3 weeks before blood withdrawal. Whole blood was used for assessment of HbA1c using an immunochemical method (Tina-quant, Roche/ Hitache, Mannheim, Germany). Glucose concentrations were measured by a glucose oxidase technique. The study protocol was approved by the Ethics Committee of the University Medical Center Utrecht.

EPC Isolation and Characterization

Peripheral blood was obtained in blood collection tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium). EPC were cultured as described[10]. Briefly, mononuclear cell fractions (MNC) were isolated from 60 ml whole blood by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO). MNC were plated at a density of 1 x 10^6 cells per cm^2 on 6-well culture plates coated with 2% gelatin (Sigma) in M199 medium supplemented with 20% FBS (Invitrogen, Breda, The Netherlands), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics and 10 U/ml heparin (Leo Pharma BV, Breda, The Netherlands). After four and seven days of culture, EPC characteristics were confirmed on the basis of morphology and by fluorescent confocal immunohistochemistry using Ulex europaeus agglutinin (UEA)-1 (Vector, Burlingame, CA, USA), a CD31 antibody (DAKO Diagnostics, Glostrup, Denmark) and DiI-labeled acetylated LDL (Molecular Probes, Leiden, The Netherlands).

Flow cytometry analyses

Quantitative determination of the percentage of cells undergoing apoptosis was determined using an Annexin V apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturers protocol. Briefly, fresh MNC and EPC, cultured for four days, were isolated as described. After the recommended washing steps, 1 x 10^6 cells were incubated for 15 min with fluorescein isothiocyanate (FITC)-conjugated Annexin V in binding buffer (BD Biosciences) in the dark. Annexin binding was measured by flow cytometry (FACScan, BD Biosciences) within 1 h and quantified using CellQuest software

(BD Biosciences). Mean levels of Annexin V binding were determined using specific monocyte and lymphocyte gates in the forward-sideward scatter plots and expressed as arbitrary units of fluorescence.

In vitro Angiogenesis Assay

The angiogenic activity of EPC conditioned media was assessed using an *in vitro* angiogenesis assay kit (Chemicon, Temecula, CA, USA) and passage two primary human umbilical vein endothelial cells (HUVEC). Conditioned media were obtained by replacing the medium of 4 day EPC cultures with serum-free endothelial cell basal medium-2 (Clonetics, Baltimore MD, USA) supplemented with EGM-2 single aliquots (growth factors like VEGF and bFGF were omitted) and cultured for an additional 30 h. EPC were counted and in subsequent experiments conditioned media were diluted to correct for EPC numbers. After 16 h, tube formation by HUVEC was measured by staining the viable cells with Calcein-AM (5μg/ml) (Molecular Probes). Total tube area was determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software (Scion Corporation, Maryland, USA) and expressed in arbitrary units.

Statistical Analysis

Statistical analysis was performed with a Student's *t* test, and results are expressed as mean ±SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPC and glycosylated hemoglobin (HbA1c). Probability values of $P<0.05$ were considered statistically significant.

Results

Patient characteristics

Subject characteristics are presented in table 1. The group of diabetic patients is representative of a type 1 diabetes population without macrovascular complications. They had a wide range of age and HbA1c. Background, preproliferative and proliferative retinopathy was present in 4, 3 and 2 patients, respectively. Microalbuminuria was present in 4 out of 20 patients.

Table 1: Subject characteristics

	Controls	Type 1 diabetes patients
n	20	20
Age (years)	39.9 ± 13.9	40.7 ± 14.8
Sex (M / F)	13 / 7	13 / 7
BMI (kg/m^2)	23.0 ± 2.6	24.7 ± 1.9
Glucose (mmol/l)	5.2 ± 1.3	7.8 ± 3.5
HbA1c (%)	-	8.3 ± 1.5
Duration of diabetes (years)	-	21.1 ± 15.2

Values are mean ± SD.

Correlation of reduced EPC number and glycosylated hemoglobin in type 1 diabetes patients

Peripheral blood MNC from type 1 diabetes patients and controls were cultured and differentiated. From four days on, next to cell clusters, EPC appeared with a typical spindle-shaped morphology[4]. EPC were further characterized by assessing the uptake of DiI-labeled acLDL, the binding of the lectin UEA-1 and the presence of the CD31 antigen, all three characteristic features of cells in the endothelial lineage. Already at day four, 80% of the attached cells stain positive for all three markers. At day seven the spindle shape morphology appears more pronounced and over 90% of the cells stain positive for all three markers. Figure 1A, shows a representative picture of a dual staining of a seven day EPC culture and Figure 1B, represents a confocal image of a representative spindle shaped cell in a seven day EPC culture, that shows uptake of DiI-labeled acLDL (red) and membrane staining with an anti-CD31 antibody (green).

Following the phenotypic characterization of our EPC cultures we assessed the number of EPC that could be obtained from four day cultures derived from the peripheral blood MNC fraction of type 1 diabetes patients and age- and gender-matched controls. We observed a significant 44% decrease in the number of EPC obtained from 60 ml peripheral blood of the

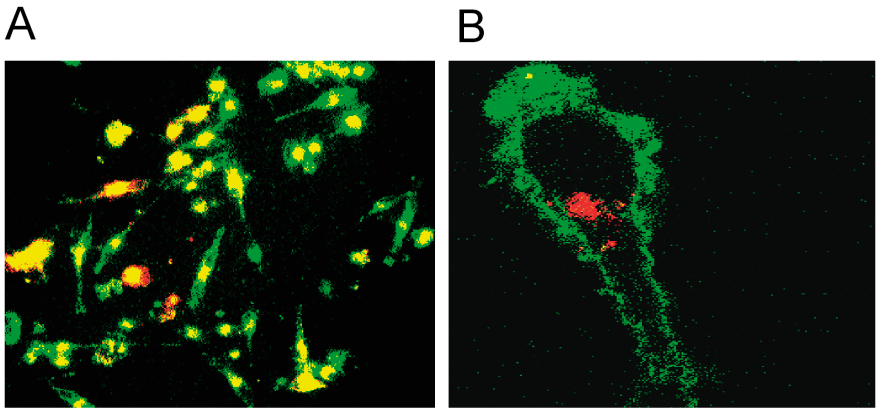

Figure 1.
EPC characterization. Fluorescent microscopy shows a representative EPC culture containing over 90% cells with a spindle-shaped morphology both staining positive for FITC labeled Ulex europaeus agglutinin and DiI-labeled acetylated LDL (A). Confocal microscopy picture showing dual staining of EPC by CD31 antibodies and uptake DiI-labeled acLDL (B).

type 1 diabetes patients compared to the non-diabetic controls ($4.3 \pm 2.3 \times 10^6$ vs. $7.8 \pm 3.3 \times 10^6$, $P<0.001$, Fig. 2A). Moreover, linear regression analyses revealed an inverse relationship between the number of EPC and the HbA1c in the patients ($R= -0.68$, $P=0.01$, Fig. 2B).

No evidence for increased apoptosis in cultured EPC from type 1 diabetes patients

To evaluate if the reduction in the number of EPC was due to apoptosis, we analyzed the binding of Annexin V to phosphatidylserine in both EPC cultured for four days and the MNC fraction they originated from. The display of phosphatidylserine in the outer leaflet of the plasma membrane of cells is considered an early marker of apoptosis. Quantitative analyses of data obtained by flow cytometry revealed no significant difference in the mean levels of Annexin V binding to EPC cultured from patients or controls when gated for viable cells (22.3 ± 10.7 and 20.4 ± 9.2 respectively, $p=0.54$), suggesting no increase in early apoptosis in these cells. Likewise, in fresh MNC fractions which were the source of our EPC cultures we also did not observe a significant increase in the mean levels of Annexin V binding of total MNC fraction in the patient group compared to that of the controls (370.9 ± 78.5 v.s. 304.7 ± 139.5, $p=0.17$).

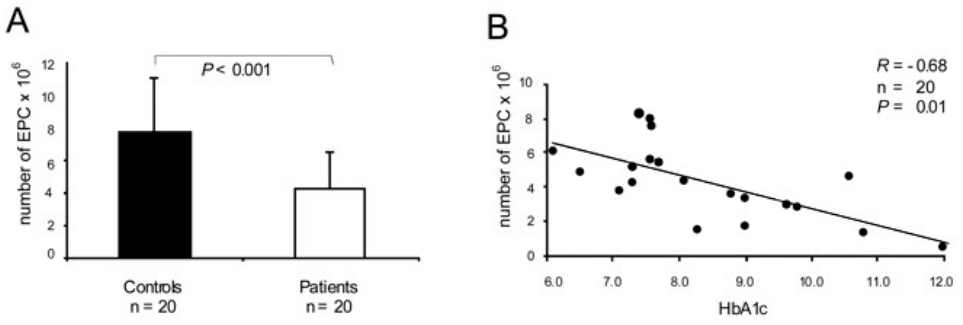

Figure 2.
MNC were isolated out of 60 ml peripheral blood from 20 controls and 20 type 1 diabetes patients. MNC were plated on gelatin-coated dishes and EPC were cultured for 4 days. The total EPC numbers were counted and a 44% reduction was seen in the type 1 diabetes patients compared to the healthy controls (A). This reduction in EPC numbers inversely correlated with glycemic control assessed by HbA1c (B).

EPCs from patients with type 1 diabetes are impaired in their potential to augment angiogenesis in vitro

EPCs are thought to augment neovascularization not only by integration of these cells into newly developing capillaries but also in a paracrine fashion through the secretion of angiogenic growth factors[5]. To investigate whether this paracrine function is affected in type 1 diabetes, we determined the angiogenic potential of EPC (day 4) conditioned medium in an *in vitro* angiogenesis model. In this angiogenesis model, the degree of tube formation of mature endothelial cells on a solid gel of matrix proteins can be evaluated. As tube formation in this assay is dependent on the presence of angiogenic stimuli we assessed the angiogenic capacity of cultured EPC. HUVEC were seeded on extracellular matrix and subjected overnight to conditioned medium of diabetic EPC (n=10) or of EPC from age-matched controls (n=10). Conditioned media of healthy controls markedly stimulated tube formation when compared to non-conditioned media (EGM -/-) demonstrating that EPC can facilitate angiogenesis in a paracrine fashion (Fig. 3). In contrast, the conditioned media of the patient EPC significantly reduced tube formation when compared to non-conditioned media suggesting that cultured EPC from type 1 diabetes patients can secrete factors that impair angiogenesis *in vitro*.

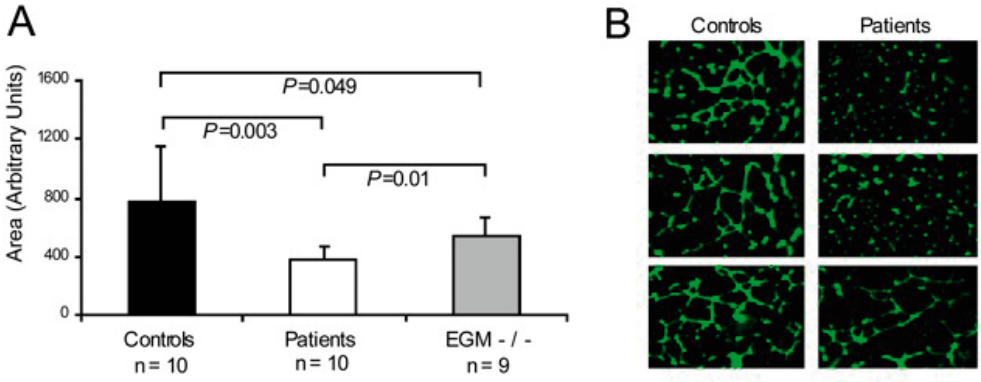

Figure 3.
Angiogenic capacity of conditioned media of EPC in an in vitro angiogenesis assay. HUVEC were plated on extra cellular matrix and subjected to EPC-conditioned media. Tubule networks were formed overnight and structures were visualized with Calcein-AM staining. Quantitative analyses of tube formation were performed using an imaging program. A significant inhibition of tube formation of patient EPC conditioned media is shown (A). Representative pictures of the stained tubule networks formed after incubation of HUVEC with the conditioned media of EPC derived from thee different patients and their age-gender matched controls (B).

Discussion

Our data support the hypothesis that EPC are dysfunctional in patients with type 1 diabetes. First, a reduced number of EPC could be cultured from the MNC fraction of type 1 diabetes patients when compared to healthy control subjects. A reduction of the number of EPC in patients with risk factors for coronary artery disease has also been reported by others[6-8]. Our data are consistent with a previous report by Schatteman *et al.*[7] who showed that $CD34^+$ cells derived from type 1 diabetes patients produced less differentiated endothelial cells than their non-diabetic derived counterparts. Here, we have extended their observation by showing that in type 1 diabetes, EPC numbers inversely correlate with HbA1c levels demonstrating that the degree of glycemic dysregulation directly affects EPC proliferation or differentiation.

As the number of circulating leukocytes is tightly regulated by the balance between proliferation and apoptosis[11] and increased apoptosis has been associated with the adverse metabolic state and oxidative stress in diabetes[12], we hypothesized that increased apoptosis

could explain the reduced EPC numbers. When we analyzed early apoptosis in EPC cultured for four days, or in the freshly isolated MNC fraction they originate from, no significant difference in the mean levels of Annexin V binding was detected in the type 1 diabetes patient group compared to the control group. These data suggest that increased apoptosis is unlikely to be involved in the reduction of the number of EPC in type 1 diabetes patients. An alternative explanation for the lower EPC counts in this study could be that the EPC precursors in the MNC fraction have an impaired capacity to adhere and/or differentiate in our culture conditions. This would represent yet another level of EPC dysfunction that is currently under investigation.

Irrespective of the underlying mechanism, a reduced number of EPC is likely to impact on vascular integrity as it was recently reported that, in healthy men, the number of EPC serve as a surrogate marker for vascular function and cumulative cardiovascular risk[9].

Having established that the number of EPC is reduced in type 1 diabetes, we determined if the function of the remaining cells was altered compared when compared to the controls. So far, only few studies addressed the subject of EPC dysfunction. It was reported that EPC isolated from patients with CAD displayed an impaired migratory response[6] and that, in type 2 diabetes, EPC adhesion to stimulated endothelial cells is impaired[8]. Although EPC enhance new vessel formation, these cells do not form the entire vessel de novo and the process always includes mature endothelial cells[13]. Recently, it has been suggested that a major function of EPC could be the secretion of angiogenic factors to activate resident mature EC[14]. Here we show that the angiogenic capacity of conditioned media from patient-derived EPC is not only reduced, but it may even contain an inhibitor for in vitro tube formation of endothelial cells.

Furthermore, our data demonstrate that type I diabetes is associated with altered EPC function and that these changes are observed even though the EPC were cultured for 4 days in a normoglycemic environment. To begin to investigate whether these functional changes are reflected in the gene expression profiles of the diabetic EPC we have performed preliminary Affymetrix DNA microarray analyses. Indeed, we observed extensive differential gene expression in patient-derived cultured EPC compared to control subjects. Interestingly, many of these alterations have been reported to be associated with diabetes mellitus in general, hyperglycemia or oxidative stress such as plasminogen activator inhibitor 1[15] and osteopontin[16]. This demonstrates that may EPC function as “bio-sensors”, translating metabolic cues into altered gene expression. How the EPC “remembers” its metabolic descent in culture is unknown and needs further investigation.

Taken together, we demonstrate that EPC cultured from type 1 diabetes patients are reduced in number and function. As a consequence, EPC dysfunction may reduce the vascular regenerative potential of this patient group and thereby contribute to the pathogenesis of vascular complications in type 1 diabetes.
Finally, the notion that EPC dysfunction exists in certain patient categories, such as type 1 diabetes, may have implications for currently explored cell-based clinical strategies to enhance tissue perfusion in patients with ischemic coronary and peripheral artery disease[17,18]. EPC or MNC isolated from these patients for autologous cell transplantation may retain their dysfunctional characteristics in vivo and as a consequence display a reduced capacity to augment therapeutic neovascularization. Therefore it could be useful to set progenitor quality criteria and perform EPC function tests (such as assays for angiogenic growth factor secretion, adhesion or migration) in order to obtain optimal cells for transplantation.

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Chapter 4

Endothelial Progenitor Cell Dysfunction in Type 1 Diabetes: another Consequence of Oxidative Stress?

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Abstract

Endothelial progenitors Cells (EPC) have been shown to contribute to neovascularization and vascular maintenance and repair in adults. Recently, the concept has evolved that EPC dysfunction, in patients at risk for cardiovascular disease, may contribute to the development of atherosclerosis and ischemic vascular disease. Particularly, patients with Diabetes Mellitus are likely to be affected by EPC dysfunction as several studies have shown a reduced number and function of EPC in patients as well as in preclinical models for type 1 diabetes. Here, we review our current understanding of EPC (dys)function in diabetes and discuss some potential mechanisms underlying their altered properties. Moreover, we provide circumstantial evidence supporting that increased oxidative stress could play a role in the development of EPC dysfunction in type I diabetes. Finally, we discuss the potential implication of our findings for EPC-based therapies and the potential impact of pharmacological interventions on the vascular regenerative capacity of EPC.

Introduction

Type 1 diabetes is not only associated with microvascular complications[1] but also with premature atherosclerosis and a reduced capacity to form collaterals vessels after an ischemic insult[2,3]. Likewise, patients with type 1 diabetes have an increased risk for the clinical consequences of these macrovascular manifestations including myocardial infarction and peripheral vascular disease[4]. Numerous studies have shown that dysfunction of the vascular endothelium plays a central role in the pathophysiology of these diseases[5]. The metabolic abnormalities that characterize diabetes, particularly hyperglycemia, provoke molecular mechanisms that have a major impact on endothelial cell function and survival. Especially, activation of protein kinase C (PKC) and increased oxidative stress can lead to endothelial cell dysfunction. Moreover, prolonged exposure of endothelial cells to these adverse conditions increases endothelial cell apoptosis and turnover. Although adjacent mature endothelial cells have the capacity to proliferate and replace these dying cells, chronic exposure to oxidative stress has been shown to lead to premature replicative senescence and limit this form of endothelial repair[6,7]. Eventually, endothelial cell death and shedding may lead to disturbances of the endothelial monolayer leaving a highly pro-atherogenic luminal surface[8,9]. Hence, the integrity of the endothelium and thus the atherogenicity of the vasculature, is likely to be determined by the balance between endothelial turnover and repair[9]. In recent years it has become clear that bone-marrow derived endothelial progenitor cells (EPC) represent an additional cellular source to rejuvenation of the damaged endothelium. EPC have been shown both in animal models and humans to contribute to neovascularization and reendothelialization indicating an essential role of these progenitor cells in the maintenance of endothelial integrity[10]. Recently, a number of studies have suggested that the classical risk factors for atherosclerosis not only affect the mature endothelium but also lead to EPC dysfunction[11,12]. This notion may not only contribute to our insight into the pathophysiology of atherosclerosis but may also have consequences for the use of progenitor cells in clinical protocols that use progenitor cell transplantation for the treatment of ischemic vascular disease. Here, we will review our current understanding of EPC dysfunction in type 1 diabetes. In particular, we will discuss the potential role of oxidative stress as an underlying cause of the dysfunction of these progenitors. Finally, we will address some potential approaches that may counteract EPC dysfunction in the clinical setting.

Endothelial progenitor cells: origins and species

It is important to appreciate that the nature of the "true" circulating EPC is poorly defined. Different populations of EPC have been studied that each may have unique properties[13]. In general, two types of EPC can be distinguished. First, circulating EPC (CEP) can be recruited from the bone marrow that are characterized by the expression of the early hematopoietic stem cell markers CD34, CD133 and the vascular endothelial-cell growth factor receptor-2 (VEGFR2)[14-16]. The EPC share these characteristics with hematopoietic stem cells and CEP may function analogous to the embryonic hemangioblast, which can give rise to both circulating blood cell lineages and vascular cells[14,17]. Cultured with endothelial cell growth factors, purified CEP can differentiate into endothelial-like cells that display a classical endothelial cell morphology and characteristics like the expression of von Willebrand factor (vWF), vascular endothelial (VE) cadherin and the capacity to take up acetylated low-density lipoprotein (acLDL). Although normally the number of CEP are limited, their levels can be markedly elevated within days after the administration of CEP mobilizing agents[18] or secondary to vascular trauma[19] or tissue-ischemia induced by myocardial infarction[20,21].

Early support for a role of bone marrow derived CEP in vascular repair in humans stems from the observation that the neointima formed on the surface of a left ventricular assist device accumulates a $CD133^+$ positive hematopoietic stem cell population that also expresses the endothelial cell marker VEGFR2[16]. In a mouse model, it was shown that bone marrow derived CEP can home to denuded arterial vessels and contribute to reendothelialization. Interestingly, statin-induced mobilization of CEP was associated with an increased rate of reendothelialization and reduced neointimal thickening[22].

A second cell type that has been shown to be involved in vascular healing can be obtained by culturing peripheral blood mononuclear cells (PB-MNC) on gelatin or fibronectin for 4 days in endothelial cell differentiation medium. These attaching cells, that are also referred to as endothelial progenitor cells (EPC), display a spindle-like morphology and also express endothelial cell markers like vWF, VEGFR2 and VE-cadherin and are usually characterized by the binding of endothelial specific lectins and the uptake of acLDL[10,13]. The large number of attaching cells that can be obtained from the PB-MNC cultures make it unlikely that all these cells are derived from the low number of circulating $CD34^+$ cells. Most likely, these EPC are derived from more abundant subpopulations present in the mononuclear cell fraction like monocytes[23-26]. When human bone marrow derived monocyte-lineage

attaching cells were intra-arterially transplanted into denuded arteries of athymic nude rats they adhered to the injured endothelium, differentiated into EC-like cells and inhibited neointimal hyperplasia[27]. Likewise, transplantation of EPC cultured from PB-MNC in rabbits led to a rapid reendothelialization of balloon-injured carotid arteries and graft segments and, again, reduced neointima deposition[28]. It is currently unclear whether the two types of EPC are related through shared developmental stages, i.e. whether a monocyte-related intermediate is a required step in the differentiation of $CD34^+$ cells into EPC.

EPC dysfunction in type I diabetes

From the above it can be concluded that EPC of different hematopoietic lineages appear to play a crucial role in the maintenance of endothelial cell integrity in injured vessels and therefore may serve an important atheroprotective function. Following these insights, it was hypothesized that impaired EPC function would predispose to endothelial cell dysfunction and its clinical manifestations including premature atherosclerosis and ischemic vascular disease. Seminal observations supporting this concept were reported by Vasa *et al.* who demonstrated that the number and function of circulating endothelial progenitor cells inversely correlated with risk factors for coronary artery disease[11]. It was shown that this concept holds true both for CD34 and VEGFR2 double positive CEP as well as for PB-MNC derived attaching EPC. Hill *et al*. extended this observation showing that, for patients at risk for CVD, there was a strong inverse correlation between the number of EC colonies that could be grown out from PB-MNC cultures and the subjects' combined Framingham risk factor score[12]. Moreover, measurements of flow-mediated brachial-artery reactivity revealed a significant relation between endothelial function and the number of progenitor cells. These reports again suggest that the quality of the endothelium may well be related to the endothelium-regenerative potential of circulating EPC.

Schatteman and colleagues were the first to report data supporting the concept of EPC dysfunction in streptozotocin-induced diabetic nude mice[29]. Using an established model for neovascularization of the ischemic hindlimb they demonstrated that, like shown before in non obese diabetic mice[30], restoration of blood flow was significantly impaired in the diabetic mice. Whereas injection of human $CD34^+$ cells, purified from the PB-MNC fraction, did not accelerate the rate of neovascularization in the healthy controls, it markedly enhanced blood-flow restoration in the diabetic mice[31]. When labeled, the $CD34^+$

cells were found to incorporate in the vasculature of the previously ischemic tissue. It was concluded that in the diabetic mice the EPC function was deficient and that this could be corrected by transplantation of exogenous human $CD34^+$ cells. These data indirectly provided evidence for deficient EPC function in experimentally diabetic mice and initiated subsequent studies to investigate the nature of the EPC dysfunction.

Diabetes-associated metabolic factors may affect EPC function at several levels, including the number of available progenitor cells with capacity to differentiate into cells of the endothelial cell lineage, their capability to adhere and migrate to sites of reendothelialization and neovascularization and their pro-angiogenic (paracrine) potential.

Effect on the number of endothelial progenitor cells

In the study by Schatteman[29] it was shown that, although the absolute numbers of $CD34^+$ cells isolated from peripheral blood from control subjects and type 1 diabetes patients did not differ significantly, the number of endothelial-like cells that formed *in vitro* from the patient $CD34^+$ cell fraction was reduced over three fold compared to the non-diabetic controls. In this study a similar analysis for type 2 diabetic patients failed to show different yields in $CD34^+$ derived EPC. However, Tepper *et al.* reported that when attaching cells were cultured from PB-MNC from human type 2 diabetics and age-matched control subjects the number of cells obtained from the patients were 48% percent lower than from healthy volunteers[32]. Likewise, we demonstrated that the number of attaching cells cultured from type 1 diabetic patients was reduced almost two fold compared to age and gender-matched control subjects[33]. In both studies this reduction was inversely related to the levels of HbA1C demonstrating that the degree of glycemic dysregulation is associated with EPC phenotype or differentiation. The most pronounced reduction of EPC numbers also was observed in a study using streptozotocin-induced diabetic mice[31]. It was found that the number of attaching cells cultured on vitronectin from BM-MNC of diabetic mice with femoral artery ligatures was five fold lower than that of control mice.

Hence, in diabetes there appears to be a reduced number of cells in MNC fractions that can differentiate into EPC *in vitro*.

Effect on the function of endothelial progenitor cells

EPC from a diabetic background have been studied for properties that are thought to be required for proper EPC function. *In vitro*, functions like adhesion to endothelial cells[32], incorporation into endothelial tubular structures[32] and paracrine release of pro-angiogenic

factors[33] were assessed and in each reported study these functions appeared significantly impaired in cells obtained from a diabetic background. As attaching EPC may be closely related to monocytes or macrophages, these results may not come as a surprise as the response of monocytes to growth factors is also impaired in diabetic patients[3].
Two reports directly asessed the function of "diabetic" progenitors cells in neovascularization in an *in vivo* model. One study investigated the effect of type 2 diabetes on the potential of exogenous stem cells to promote skin wound vascularization and healing[34]. Bone marrow stem cells from nondiabetic and diabetic Leprdb mice were injected underneath experimentally induced skin wounds. It was shown that, in contrast to nondiabetic stem cells, diabetic stem cell containing fractions not only failed to enhance but even inhibited wound vascularization. A second study reported that transplanted diabetic EPC, obtained from BM-MNC fractions of streptozotocin-induced mice, were markedly impaired in their capacity to enhance ischemia-induced neovascularization assessed by the ischemic/nonischemic angiographic score, capillary number and blood flow recovery. Taken together, evidence is accumulating that, in diabetes, the number and function of EPC pools are reduced and therefore may be involved in the pathogenesis of both vascular complications.

Potential role of oxidative stress on EPC dysfunction in diabetes

Then, what molecular mechanisms may cause this reduction in EPC capacity in diabetes? It is clear that the answer to this simple question will be complex and dependent on the particular risk factors present and type of diabetes that affects individual patients. In type 1 diabetes, chronic hyperglycemia appears to be the major initiator of vascular complications through the increased production of reactive oxygen species (ROS) by the vascular endothelium[5,35]. Endothelial cells are particularly sensitive to hyperglycemia as they, unlike most cell types, are not capable of down regulating glucose uptake in high ambient glucose concentrations[36]. Hyperglycemia can lead to elevated ROS production in the endothelial cells via a number of enzymatic systems including the mitochondrial electron transport chain, activation of NADPH oxidase and uncoupling of endothelial nitric oxide synthase (eNOS)[37]. Although the endothelial cells are equipped with potent antioxidant systems, sustained production of ROS in chronic hyperglycemia can exhaust these protective mechanisms and lead to a state of "oxidative stress"[38]. This condition is associated with

endothelial cell dysfunction, a proinflammatory endothelial phenotype that is characterized by a reduced bioavailability of NO.

Oxidative stress and EPC mobilization

Another landmark study performed in the laboratory of Stephanie Dimmeler provided a possible explanation for the reduced mobilization of EPC in patients with cardiovascular disease[39]. Using eNOS knock-out mice they demonstated that NO expressed by bone marrow stromal cells plays an essential role in vascular endothelial growth factor (VEGF)-induced mobilization of CEP ($CD34^+/VEGFR2^+$) from the bone marrow stroma to the vascular compartment. As endothelial cell dysfunction and impaired NO bioavailability is the hallmark of most cardiovascular risk factors these data support the hypothesis that CEP mobilization is impaired secondary to oxidative stress. Hyperglycemia also decreases endothelium-derived NO both *in vitro*[5] and during hyperglycemic clamping in healthy subjects[40]. It therefore seems likely that also in diabetes a reduced bioavailability of NO in the bone marrow stroma is involved in the reduced mobilization of EPC.

Oxidative stress and EPC function

Given the central role of oxidative stress in type 1 diabetes, oxidative stress or altered redox signaling may also directly affect the survival, differentiation and function of EPC. Although little data on CEP function have been reported, a number of papers may provide indirect evidence for a role of redox signaling in the fate and function of MNC-derived EPC (attaching cells). Given the fact that these cells are thought to function in sites of ischemia or reperfused tissue that can be characterized as an inflammatory, high oxidative stress environment[13], Dernberg *et al.* investigated the anti-oxidative systems of cultured EPC[41]. They demonstrated that, compared to mature umbilical vein endothelial cells (HUVEC), EPC exhibited a significantly lower basal ROS concentration and a relative high expression of the intracellular anti-oxidative enzymes catalase, glutathione peroxidase and manganese superoxide dismutase (MnSOD). Incubation of HUVEC with H_2O_2 increased ROS production up to 4-fold and induced apoptosis. In contrast, H_2O_2 hardly affected ROS production and apoptosis in the EPC demonstrating that EPC display a reduced sensitivity towards ROS-induced cell death. Combined inhibition of the antioxidant enzymes increased ROS levels in the EPC and impaired EPC survival and migration. He *et al.* demonstrated a critical role for MnSOD in protecting EPC from cytotoxicity induced by the naphtoquinolinedione LY83583, a generator of intracellular superoxide[42]. LY83583

inhibited *in vitro* tube formation by mature endothelial cells but not by EPC. These data suggest that although EPC are relatively resistant to oxidant stress, elevation of ROS production can affect survival and function, most likely by affecting redox-sensitive signaling pathways, such as the NF-κB pathway[43].

Potential mechanisms of diabetes-associated ROS production by EPC

The question remains how in a diabetic environment oxidative stress would be elevated in these EPC. It is unclear to what extend mechanisms that are known to function in mature endothelial cells can be translated to EPC. For instance, can cultured EPC take-up glucose in the same apparently "uncontrolled" way as mature endothelial cells[36]. Nevertheless, hyperglycemia induced activation of PKC and its downstream effects on e.g. activation of NADPH oxidase could be a potential mechanism for elevation of ROS in EPC. Also, hyperglycemia-associated formation of extracellular and intracellular advanced glycation end products (AGE) may affect the redox state of the cells. AGEs are the products of nonenzymatic glycation/oxidation of proteins and lipids and have been regarded as one of the main mechanisms responsible for vascular damage in patients with diabetes[44]. It was shown that, in streptozotocin-treated diabetic mice, blockade of AGE formation restores ischemia induced angiogenesis[45]. AGEs are signal transduction ligands for Receptor for AGE (RAGE) that, upon AGE binding, trigger the generation of ROS and the proinflammatory NFκB pathway via activation of NADPH oxidase[46]. RAGE is present on monocytes[47] and also EPC cultures from PB-MNC (unpublished data our laboratory).

As hyperglycemia in type 1 diabetes is associated with activation of the renin angiotensin system[48], angiotensin II signaling could comprise a third route to ROS production in EPC. Ramipril is an ACE inhibitor that is used to reduce RAAS activation in patients with stable coronary artery disease. A recent study showed that increased numbers of EPC could be cultured from ramipril treated patients with stable coronary artery disease and that the ACE inhibition resulted in improved functional properties like adhesion, proliferation, migration and an *in vitro* vasculogenesis assay[49]. These results show that EPC are sensitive to angiotensin II signaling and that this could indeed impact on number and function.

EPC from type 1 diabetes patients up regulate genes associated with oxidative stress

To further investigate whether EPC in type 1 diabetes are altered in function due to the adverse metabolic environment, we analyzed whether changes in gene expression could be observed between the patient and control EPC. Therefore, we compared the gene expression profiles of pooled RNA isolated from cultured EPC obtained from five type 1 diabetes patients (age 34.2 ± 10 years) and five age-and gender-matched controls (age 33.9 ± 7.7 years) using Affymetrix high-density oligonucleotide microarray analysis (summarized in table 1). In the diabetic EPC, out of 12,600 gene transcripts tested, we observed significant up- and down regulation of 472 and 360 genes respectively. Among the major differentially expressed genes we observed a striking number of genes that have been have been reported to be associated with diabetes mellitus in general, with hyperglycemia, oxidative stress or AGEs, both in a clinical setting (e.g. osteopontin[50], plasminogen activator inhibitor 1[51], thombomodulin[52] and type IV collagen[53]) as well as in animal models (matrix metalloproteinase 1[54], lectin-like oxidized-LDL receptor[55],

Table 1. Differential expression of diabetes associated genes in cultured EPC from type 1 diabetes patients.

Protein name	Gene name	Genbank	Fold Change	P-value	Category
Osteopontin	SPP1	J04765	19.7	< 0.00001	D
Plasminogen activator inhibitor 1	PAI1	J03764	17.1	< 0.00001	D
Alpha-2 type IV collagen	COL4A2	M33653	11.3	0.0002	D
Lectin-like oxidized-LDL receptor	LOX1	AF079167	8.0	< 0.00001	D
Fructose-1,6,-biphosphatase	FBP1	U21931	3.7	< 0.00001	D
Thombomodulin	THBD	J02973	3.5	< 0.00001	D
Cystatin A	CSTA	AA570193	2.3	0.00005	D
Heat shock protein	HSP27	Z23090	1.9	0.0004	D/O
CD11b, Complement receptor 3	MAC1	J03925	1.3	0.0010	D
Matrix metalloproteinase 1	MMP1	Z48481	-1,9	< 0.00001	D
VEGF	VEGF	M97863	-2.5	0.0006	D
MHC class II HLA-DR2-Dw12	MHC2	M16276	-2.8	< 0.00001	D
GTP cyclohydrolase I	GCH1	U19523	-4.3	0.00001	D
Macrophage scavenger receptor 1	MSR1	D13264	19.7	0.00001	O
Hepatic dihydrodiol dehydrogenase	AKRC1	U05861	13.9	< 0.00001	O
Glutathione S-transferase A4-4	GSTA4	AF025887	8.6	0.0002	O
Peroxiredoxin 2	PRDX2	L19185	2.5	0.00003	O
A2b adenosine receptor	ADORA2B	X68487	2.5	< 0.00001	O
P8 protein	P8	W47047	2.3	0.0002	O
Superoxide dismutase 1	SOD1	X02317	1.3	0.0008	O

Fold changes were calculated with control values as baseline comparison file. P -values express the significance of the fold changes as calculated by Affymetrix Microarray Suite 5.0 software. Categories represent genes whose products were reported to be induced or repressed in hyperglycemia or type 1 diabetes (D) or as a consequence of increased oxidative stress (O).

fructose-1,6,-biphosphatase[56] and GTP cyclohydrolase I[57]). Our data demonstrate that EPC function as "bio-sensors", translating metabolic cues into altered gene expression and support the hypothesis that dysfunction of the EPC in type 1 diabetes may be secondary to elevated oxidative stress.

Conclusions and implications

Recent data shows that the vascular regenerative potential of patients with diabetes may be impaired as a consequence of reduced number and function of circulating progenitor cells that can support endothelial maintenance and ischemia-induced neovascularisation. Although direct evidence is lacking, indirect evidence supports a role for oxidative stress in the diabetes-associated EPC dysfunction. Notably, our DNA microarray analysis suggests that EPC cultured from PB-MNC fraction from type 1 diabetes patients display a pro-inflammatory phenotype. The implication of these findings is that autologous transplantation of progenitor cells that are affected by risk factors, such as high glucose, may not only be hampered by a dysfunctional nature of these cells[58] but in fact may stimulate pro-atherogenic mechanisms such as monocyte recruitment or vascular smooth muscle cell proliferation. Interestingly, a recent study, in which G-CSF mobilized vascular progenitor cells were infused into patients with mycoardial infarction to improve cardiac function, showed enhanced in-stent restenosis which led to a premature termination of the trial[59].

A current concept is therefore that autologous progenitor cell therapy, in patients with cardiovascular risk factors, probably should be accompanied by drug therapy that modulates the dysfunctional and adverse phenotype of these cells. First, the particular risk factor should be carefully treated through conventional approaches. In diabetes this would mean that an optimal control of hyperglycemia should be pursued for some time in advance of the isolation of progenitor cell fractions. Second, an adjunctive therapy could be used to improve EPC function. For instance, HMG-CoA reductase inhibitors have been shown to increase the number of circulating EPC both in animal models[60,61] as well as in patients with stable coronary artery disease[62]. In these studies, statins were also shown to improve functional aspects of the studied EPC populations *in vitro*, like proliferation, migration[60,62], chemotaxis[60], and adhesion[22]. Statin treatment also augmented corneal neovascularization

in mice[60] and reendothelialization after vascular injury in rats[22] and in both models the contribution of BM-derived EPC to these effects was increased.
In coronary artery sections it was shown that statins can reduce glucose induced ROS production by the endothelium through the inhibition of GTPase mediated activation of the NADPH subunit p22Phox[63]. This observation together with the fact that EPC can respond to statins would suggest that inhibition of HMG-CoA reductase may also counteract the adverse EPC phenotype observed in EPC cultured from the PB-MNC of type 1 diabetes patients or *in vivo*.
Likewise, pharmacologic intervention in angiotensin II signaling by ACE inhibitors or angiotensin receptor 1 antagonist may also proof beneficial to EPC number and function. Whatever future therapeutic strategy will prove effective, it seem most likely that redox signaling will be one of its targets.

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Chapter 5

Angiogenic murine endothelial progenitor cells are derived from a myeloid bone marrow fraction and can be identified by endothelial NO synthase expression

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Abstract

Objective

Endothelial progenitor cells (EPC) contribute to postnatal neovascularization and are therefore of great interest for autologous cell therapies to treat ischemic vascular disease. However, the origin and functional properties of these EPC are still in debate.

Methods and results

Here, *ex vivo* expanded murine EPC were characterized in terms of phenotype, lineage potential, differentiation from bone marrow (BM) precursors and their functional properties using eNOS-GFP transgenic mice. Despite high phenotypic overlap with macrophages and dendritic cells, EPC displayed unique eNOS expression, endothelial lineage potential in colony assays and angiogenic characteristics but also immunological properties such as IL12p70 production and low levels of T cell stimulation. The majority of EPC developed from an immature, $CD31^{+}Ly6C^{+}$ myeloid progenitor fraction in the BM. Addition of myeloid growth factors such as M-CSF and GM-CSF stimulated the expansion of spleen-derived EPC, however not BM-derived EPC.

Conclusion

The close relationship between EPC and other myeloid lineages may add to the complexity of using them in cell therapy. Our mouse model could be a highly useful tool to characterize EPC functionally and phenotypically, to explore the origin and optimize the isolation of EPC fractions for therapeutic neovascularization.

Introduction

Human peripheral blood contains bone marrow (BM)-derived progenitor cells with angiogenic properties[1-3]. These cells have the potential to differentiate towards endothelial cells and are therefore named endothelial progenitor cells (EPC). Transplantation of EPC has been shown to be effective in animal models for re-endothelialization[4,5] and adult neovascularization[6,7] as well as in human patient studies aimed to enhance myocardial regeneration after acute myocardial infarction[7]. Although EPC are used in clinical trials, the exact phenotypic and lineage-/differentiation parameters of *ex vivo* expanded EPC are poorly defined and it is not clear which cell populations will be most effective in repair studies. EPC can be derived from $CD34^+$ as well as $CD34^-$ or $CD34^{low}$ cells and can be isolated and expanded *ex vivo* using BM aspirates and peripheral blood $CD14^+$ mononuclear cell fractions[8-12]. In many studies, EPC are characterized by their adhesive spindle-like morphology, staining with the endothelial cell binding lectin Ulex europaeus agglutinin (Ulex) and the capacity to endocytose acetylated LDL[2,11]. Although this may generally suffice for EPC studies dealing with EPC obtained from healthy animal models or humans, the different culture conditions and sources used may lead to a large heterogeneity and functionally suboptimal EPC populations[13,14]. It has even been suggested that transplantation of certain cell fractions may contribute to adverse side effects[15]. Clinical studies demonstrated that in patients suffering from diabetes and hypertension, the number of circulating EPC is severely decreased and the cells are dysfunctional[16-19]. This altered phenotype of EPC could contribute to and might even endow the progression of the pathogenesis of ischemic vascular disease in these patients.

It has been shown that cells from the myeloid lineage, e.g. EPC, show a wide phenotypic overlap[20] and as we demonstrate here that Ulex and the uptake of acLDL, among other often used endothelial markers, are not specific for EPC. Therefore discrimination is difficult between EPC and other myeloid cells such as dendritic cells (DC) and macrophages (Mph) which are also in close contact with the vascular system[21,22]. Myeloid progenitor cells exhibit a very high plasticity and under different circumstances a precursor cell can be skewed towards alternative differentiation directions[14,23-26].

To characterize better the nature of the angiogenic myeloid cell (EPC) compared to other myeloid cells and mature EC, we first performed a detailed comparative phenotypic and functional analysis of cells stimulated to differentiate into EPC, DC or Mph starting from

the same progenitor cell populations. Secondly, we have employed a transgenic mouse model expressing endothelial Nitric Oxide Synthase (eNOS) fused to green fluorescent protein (GFP)[27]. The expression of the transgene is driven by the native human eNOS promoter and the transgenic mice show an endothelium specific expression pattern in many different organs. This transgenic mouse model therefore is expected to precisely distinguish cells from the EC lineage from other myeloid cells.

Materials and methods

Animals.

C57BL/6J, and FVB wildtype mice 6-22 wk of age were obtained from Harlan (Horst, The Netherlands). eNOS-GFP transgenic mice were generated as described previously[27] and bred at the Central Animal Department of the Erasmus MC (Rotterdam, The Netherlands) under the institutional guidelines.

Isolation and differentiation of murine EPC, DC and Mph

Single-cell BM suspensions, were prepared by flushing femora and tibiae with medium. Mononuclear cells were isolated by ficoll density gradient centrifugation. Unless mentioned otherwise, EPC cultures were plated at a density of 1 x 10^6 cells per cm^2 on 24-well plates (Nunc) coated with 10 µg/ml fibronectin (Sigma) and cultured up to 7 days in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV). Several different culture conditions were tested for optimal EPC culture condition[14,16,18]. Two different coating materials fibronectin (10 µg/ml) and gelatin (2%, Sigma) were tested with either BPE-containing medium described above[18] or endothelial basal medium (EBM) (Clonetics) supplemented with endothelial growth medium SingleQuots and 20% FCS[16] with or without extra addition of Vascular Endothelial Growth Factor (VEGF, 100 ng/ml, Peprotech)[14]. Experiments using eNOS-GFP tg BM revealed that the combination of BPE containing medium and fibronectin-coated surfaces are the most optimal conditions for generating the highest number of GFP^+ EPC after 7 days of culture. These EPC culture conditions are used throughout the study, unless mentioned otherwise.

Mph and DC were cultured from total BM isolates in 10 cm Petri dishes (BD Biosciences) at a density of 2 x10^6. For DC culture we used 20 ng/ml recombinant murine GM-CSF

(Biosource) and for Mph cultures we used 10 ng/ml recombinant murine M-CSF (Peprotech). For activation, EPC, Mph and DC were incubated with 50 ng/ml Lipopolysaccharide (LPS, Sigma) at day 6. After overnight incubation at 37ºC, the culture supernatants were collected and frozen for cytokine measurements and the cells were harvested for mixed leukocyte reactions (MLR).

An often used mouse brain derived mature endothelial cell line; bEnd3 was used for phenotypic comparisons as well as lung EC isolated from eNOS-GFP transgenic mice. The lung EC were harvested by collagenase treatment of murine lung tissue. Single cell suspensions were cultured with BPE containing medium (as described) on fibronectin coated flasks and the cells were cultured for 4-6 passages.

HUVEC were isolated by trypsin treatment of umbilical cords and cultured with BPE containing medium for 3 passages.

Antibodies and conjugates for cell sorting, flow cytometric and immunohistochemical analysis

Undiluted culture supernatants of the hybridomas ER-MP12 (anti-CD31), ER-MP20 (anti-Ly-6C), F4/80, MECA-20 (mouse endothelial cell antigen-20) were directly used for staining[28]. Phycoerythrin- (PE-) labeled anti-CD11c, biotinylated anti-MHC class II and anti-CD14 were purchased from BD Biosciences. Secondary antibodies FITC- or PE-labeled goat anti-rat IgG (GαR-FITC or GαR-PE) were purchased from Caltag Laboratories and BD Biosciences respectively. ER-MP12 was purified and biotinylated and ER-MP20 was labeled with FITC conjugate[29]. Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Biosciences). Directly PE-labeled murine antibodies directed to KDR, Sca-1, ckit and CD34 were purchased from BD Biosciences as well as the directly labeled PE isotype controls. Unlabeled MECA-32 and VE-cadherin (BD Biosciences) and isotype controls were labeled with PE-labeled goat anti-rat IgG PE (BD Biosciences) and Flt-1 (Santa Cruz) and its isotype control was labeled with PE-labeled goat-anti Rabbit IgG (BD Biosciences). For lectin staining, cells were stained with rhodamine labeled Bandeiraea Simplicifolia lectin (BS-1 lectin 10μg/ml, Vector labs). This labeling was performed in cell suspension for flowcytometric analyses and for immunohistochemical stainings. To that end, cells were attached to fibronectin coated-glass slides and incubated for half an hour and fixed with 3% paraformaldehyde. To visualize each cell nucleus, Hoechst (33258, Invitrogen) staining was performed according to the manufacturers' protocol. To measure the uptake of DiI-labeled acetylated LDL (Molecular

Probes) with flow cytometric analysis, cells were incubated (2.4 µg/ml) for 2 hours at 37°C and counterstained with Ulex europaeus agglutinin (UEA)-1 (10 µg/ml, Vector), further referred to as Ulex, for 1 hour. Flowcytometric analyses expression were assessed using FACScan (BD Biosciences) and analyses were quantified using CellQuest software (BD Biosciences).

Cell sorting

For cell sorting, BM-derived cells were labeled with ER-MP12^{bio} (anti-CD31) and ER-MP20^{fitc} (anti-Ly-6C)[29]. Before sorting (FACSVantage; BD Biosciences), cell suspensions were filtered over a 30-µm pore size sieve (Polymon PES) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-analyzing sorted samples, and purity exceeded 95%.

Cytokine detection

IL-10 and IL-6 ELISA kit (Biosource), and IL-12p40 and IL-12p70 ELISA kits (R&D) were used according to the manufacturers protocol.

MLR assay

Mixed leukocyte reactions (MLR) were done with allogeneic T cells from C57/Bl6 splenocytes. Cells were incubated with Abs recognizing CD11b, CD45 and MHCII and anti-rat IgG microbeads. Naïve T cells were obtained by negative selection using a magnetic cell sorter. Stimulated (LPS) or non-stimulated DC, Mph and EPC were irradiated sub-lethally. T cells (1.5 x 10^5 cells/well) were added to varying concentrations of stimulator cells depending on the desired stimulator-responder cell ratio. Proliferation of T cells was measured after 4 days by uptake of ^{3}H-thymidine (1 µCi/well, DuPont-NEN) and expressed as counts per minute (cpm).

In vitro angiogenesis assay

Conditioned media were obtained by replacing the medium of 6-day EPC cultures with serum-free EC basal medium-2 (Clonetics) supplemented with EGM-2 single aliquots (no vascular endothelial growth factor and basic fibroblast growth factor) and culturing the cells for an additional 16-20 h. EPC were counted and conditioned media were diluted to correct for cell numbers. After 14 h, tube formation by HUVECs was measured by staining the viable cells with Calcein-AM (5 µg/ml) (Molecular Probes). For quantification, total tube

area was determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software (Scion Corporation) and expressed in arbitrary units. To see the ability of EPC, DC and Mph to incorporate and/or participate in the formation of vessel-like structures, HUVEC were stained with PKH26 (Sigma), a red fluorescent cell linker dye, according to the manufacturer's protocol. EPC, DC and Mph were stained with Calcein-AM and the labeled cells were applied on the *in vitro* angiogenesis assay kit in a ratio of 1:4 (EPC: HUVEC). After 14 hours, incorporation/participation of EPC, DC and Mph were evaluated with a confocal microscope (Carl Zeiss) using z-stack images.

Endocytosis assay.

Uptake of dextran-FITC was done at 37°C and 4°C (negative controls) for 30 min. Cells were carefully washed and uptake of dextran was measured by flowcytometric analyses.

Real-Time Quantitative-PCR (RQ-PCR)

Total RNA preparations sorted cell populations were performed using the RNeasy kit (Qiagen) and the integrity of RNA was checked before further use (Bioanalyzer, Agilent). mRNA expression of human eNOS and two murine normalization genes (Actin and GAPDH) were analyzed using quantitative RT-PCR. cDNA was synthesized from total RNA samples using standard cDNA synthesis reagents and a 1:1 mixture of oligo $dT_{(12-18)}$ primers and random hexamer primers (Invitrogen). Quantitative analyses of the synthesized cDNA were performed with use of SYBR green I (Molecular Probes) in real-time PCR (Amplitaq Gold,Applied Biosystems), using an iCycler Thermal cycler (Biorad). Gene specific primer combinations were generated with Oligo Explorer (Gene link) and synthesized (Isogen). eNOS forward primer: GGCTCTCACCTTCTTCCTG, reverse primer: ACCACTTCCACTCCTCGTAG. For normalization genes, primer sets GAPDH forward: ACTCCCACTCTTCCACCTTC reverse: CACCACCCTGTTGCTGTAG and also actin forward:GACTTCGAGCAGGAGATG reverse:GGTACCACCAGACAGCAC were used.

Samples were analyzed in triplicate and threshold cycle numbers and their SD were calculated using icycler v3.0a analysis software (Biorad) and further used to calculate expression ratio's of the different samples in relation to both normalization genes.

EPC colony formation (CFU-EC)

To assess the property of EPC to differentiate to EC and to proliferate we have used an established CFU-EC assay (Endocult, StemCell Technologies). After 48 hours, non-adherent cells were collected and replated in fibronectin-coated 24-wells plates. After 3 days, GFP^+ colonies were detected using fluorescence microscopy.

Statistical analysis.

Results are expressed as mean ± SD. Probability values of $P < 0.05$ were considered statistically significant (Student t-test).

Results

Morphologic and Phenotypic comparison of EPC, DC and Mph derived from bone marrow.

Due to the high phenotypic overlap of EPC with other cells of the myeloid lineage it is important to define the criteria that characterize EPC in more detail. To that end, we first investigated morphological and functional differences between EPC, DC and Mph cultures obtained from mouse BM. In figure 1A (upper panel) the distinct morphology of the different cells at day 7 is shown. EPC showed typical spindle-shaped morphology, DC displayed long extended dendrites or veils and Mph were more rounded-up and attaching. EPC were capable of binding Ulex and taking up acLDL to the same extent as Mph. DC stained for Ulex but hardly took up acLDL particles. Therefore, Ulex staining combined with the uptake of acLDL are not appropriate markers restricted to EPC.

Next we determined the expression of surface markers to further characterize EPC (CD31, MECA-20, MECA-32, BS-1 lectin, Flt-1, c-kit, Sca-1, KDR, VE-Cadherin and CD14), DC (CD11c and MHCII) and Mph (F4/80, CD11b) (Fig 1B, Fig I). EPC displayed a higher expression of MECA-20, CD14 and CD31, in comparison to Mph and DC (Fig 1B). EPC and Mph showed a lower expression level of CD11c and MHCII when compared to DC. The Flt-1 receptor is highly up regulated in total population of the EPC but also on a small population of DC (Fig I). MECA-32 antibody showed expression on a very small subset of the EPC and no expression on Mph and DC, while MECA-20 (also reported as EC specific[30,31]) does show a higher expression on the EPC fraction when compared to DC an

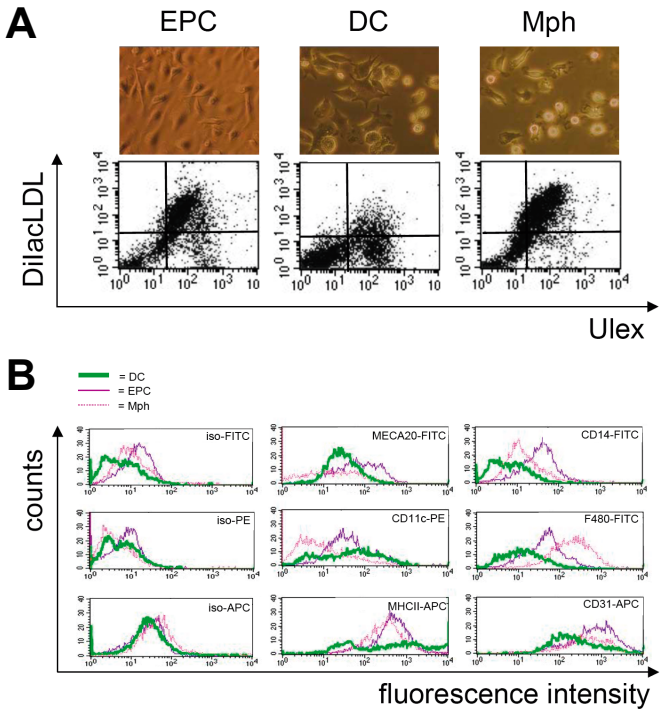

Fig 1. Morphologic and phenotypic characterization of murine EPC compared to DC and Mph.

(a) phase contrast microscopic morphological appearance (upper panel) and flow cytometric analyses of the ability of the different cells to bind the lectin Ulex and to take up DiI-labeled acLDL particles (lower panel). The analyses were plotted and non-stained cells served as negative controls (see quadrilles) (b) representative flow cytometric analyses of different lineage specific antigens. The thick green lines represent the DC, the thin dark line the EPC and the pink-dashed line the Mph. Cells were cultured for 7 days starting with total BM under optimized culture conditions as described in Methods.

Mph. Thus, a unique marker specifically defining EPC was lacking. At best, EPC could be characterized and distinguished from DC and Mph as spindle-shaped cells that were CD31^{hi}, MECA-20^{hi}, Flt ^{hi} and F4/80^{lo}.

Functional comparison of EPC, DC and Mph derived from bone marrow.

Conditioned medium (CM) of EPC, Mph and DC was tested for supporting formation of tube-like structures in an *in-vitro* angiogenesis assay. While conditioned medium of DC and Mph hardly showed any induction of tube-like structures, EPC CM significantly augmented the formation of tube-like structures (Fig IIA). Secondly, using confocal microscopy, we compared the three different cell types for their ability to incorporate into and/or to

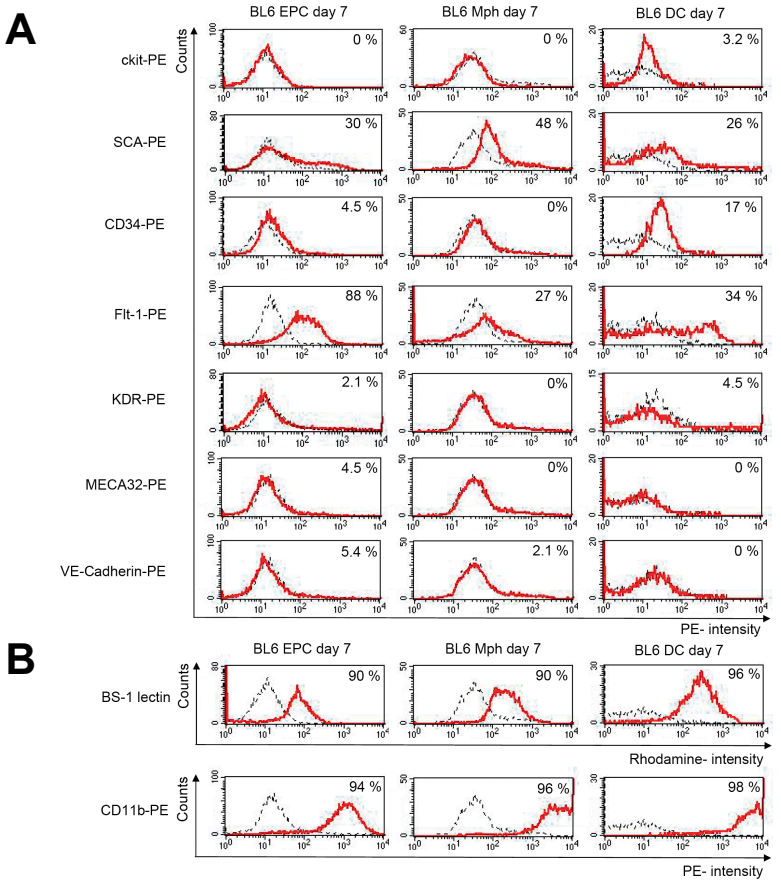

Fig I. Additional phenotypical characterization of EPC, Mph and DC cultured for 7 days.
C57BL/6J BM was cultured under the appropriate conditions for all three cell types. Cells were harvested and stained for flow cytometric analysis with various antibodies. All antibody stainings (filled lines) were corrected with isotype controls (dotted line) and representative histograms are shown (5 experiments performed). Percentage of positive cells was calculated and staining of isotype controls were subtracted. No distinctive marker for EPC could be identified using these antibodies. Figure 1B, shows extra histograms for BS-1 lectin binding and for a myeloid marker CD11b.

participate in the formation of tube-like structures. While DC, Mph and EPC all attached to the protrusions of the EC, only EPC were able to specifically adhere to, and line up in tube-like structures (Fig IIB, arrows). Thus, only EPC and not DC or Mph display genuine pro-angiogenic properties by both factor production and participation in tube formation.

Next, we addressed functional properties specific for Mph and DC. Macrophages endocytose to clear the body of pathogens, while DC mainly use their endocytic properties to present antigens to T lymphocytes. Mph displayed a high endocytic capacity (fig IIC),

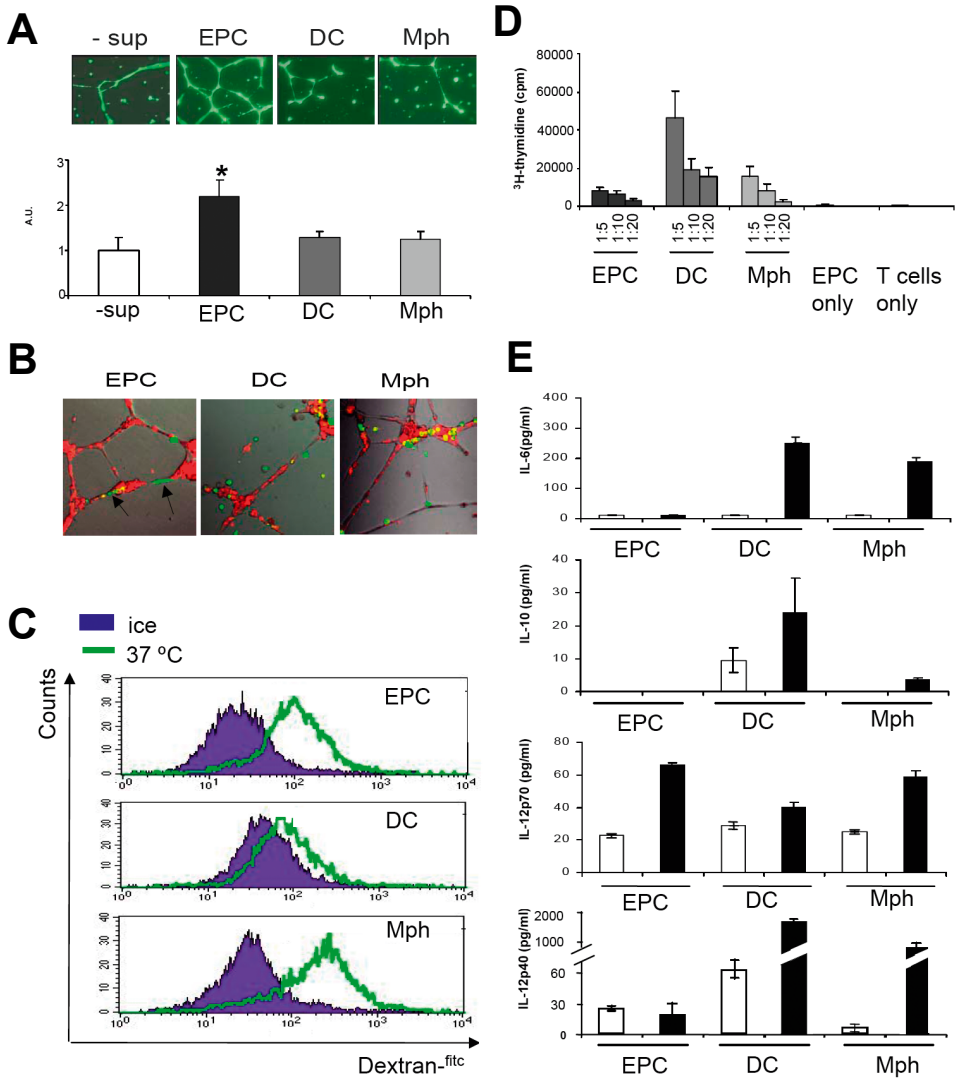

Fig II. Functional characterization of murine EPC compared to DC and Mph.

(a) *In vitro* angiogenesis assay. Representative pictures of calcein-labeled tubular structures formed by HUVEC under conditioned media produced by EPC, DC or Mph. Quantitative analyses of the angiogenic capacity of the conditioned media were performed. Data are shown relative to non-conditioned media as control (-sup), which was set at 1.0. Only EPC were able to significantly (* $P < 0.05$) stimulate angiogenesis. (b) Incorporation into vessel like structures *in vitro*. The ability of EPC, DC and Mph to incorporate and/or participate in *in vitro* formed vessel structures was measured by using an *in vitro* angiogenesis setting. Thereby HUVEC were stained with PKH (red) and EPC, Mph and DC cultured for 7 days were stained with Calcein-AM (green). Cells were visualized by confocal microscopy and representative pictures show tubular EPC (arrowheads) while the morphology of DC and Mph did not change. (c) Endocytosis assay. The capacity of EPC, DC and Mph to take up large dextran-FITC molecules was examined on ice (filled blue plot; control) and at 37°C (green line). EPC

and Mph efficiently phagocytozed dextran, whereas DC showed less capacity above control levels (ice). (d) Mixed lymphocyte reaction. The proliferation of T-lymphocytes was measured by incorporation of 3H-thymidine at day 4 after initial contact with LPS-stimulated EPC, DC or Mph. The 3H-thymidine incorporation was counted and plotted in cpm. LPS stimulated EPC induced T cell proliferation to ~20% of the levels found by adding equal cell numbers of DC, but clearly showed stimulatory capacity in MLR compared to EPC only or T cell only. Values represent the means of triplicate measurements ± SD. This stimulatory capacity was slightly lower than the levels of LPS stimulated Mph. (e) ELISA's were performed for IL6, IL10, IL12p70 and IL12p40 with conditioned media of non-stimulated (light bars) as well as LPS-stimulated (dark bars) EPC, DC or Mph. Values represent the means of triplicate measurements ± SD. The only detectable cytokines produced by EPC were IL12p70 and IL12p40. LPS stimulation increased IL12p70 production, but not IL12p40 production.

but EPC also showed an almost similar capacity to take up the dextran molecules. DC barely showed endocytic capabilities above control values. A typical feature of DC is antigen presentation to, and cytokine activation of, naïve T lymphocytes for instance in a mixed lymphocyte reaction (MLR). As expected, mature/activated DC were able to trigger T cell proliferation. EPC activated by LPS could do this as well, but to a lesser extent (Fig IID). Unstimulated EPC hardly induced T cell proliferation (data not shown).

To evaluate the cytokine profile of EPC compared to that of DC and Mph we measured IL-6, IL-10, IL-12p70, IL-12p40 in CM of non-stimulated and LPS-stimulated cells. Although DC and Mph were capable of producing all four cytokines, EPC secreted detectable levels of IL-12p70 and IL-12p40 only. IL-12p70 was produced by the EPC to a similar level as Mph and DC and LPS stimulation of the EPC strongly enhanced this IL-12p70 production (Fig IIE). IL-12p40 was produced by EPC, although to a lower extent than by DC and LPS stimulated Mph. IL-12p70 has been shown to be an active subunit of IL-12, which can regulate T cell-mediated immune responses by promoting Th1 development. It is striking that IL-12p70 is the predominant IL-12 subtype produced by EPC. Concluding, only EPC have the capacity to induce *in vitro* angiogenesis, yet they share with DC and Mph the capability to endocytose and are also able to act, to some extend, as APC with IL-12 producing capacity.

Tracking EPC differentiation by using the endothelial specific marker eNOS coupled to GFP.

Since there was a considerable phenotypic and also some functional overlap between the EPC, DC and Mph, we aimed to specifically track BM derived cells differentiating towards the endothelial lineage (EPC). Therefore a transgenic mouse model was used in which the mice show an endothelium-specific GFP expression pattern[27]. When BM of eNOS-GFP transgenic C57Bl/6J mice was harvested (day 0) a small population of cells (about 0.05%

of total cells) expressed GFP in the transgenic mice (tg) which is not present in control BM isolates (day 0) of wild type mice (wt) (fig.2A). At day 7 of culture under EPC culture conditions, about 15% (n=6, representative experiment shown) of the attached cells were GFP^+ in the tg EPC. There is a high autofluorescent background of cells in the EPC cultures at day 7 in both FL1 and FL2 channels, which is seen in transgenic BM cultures as well as wildtype BM cultures.

When EPC cultures were flow-sorted at day 7 and the GFP^- and GFP^+ populations were replated separately at the same concentrations, only the GFP^+ fraction (by definition expressing eNOS, fig IV) cells displayed the typical EPC morphology of spindle-shaped

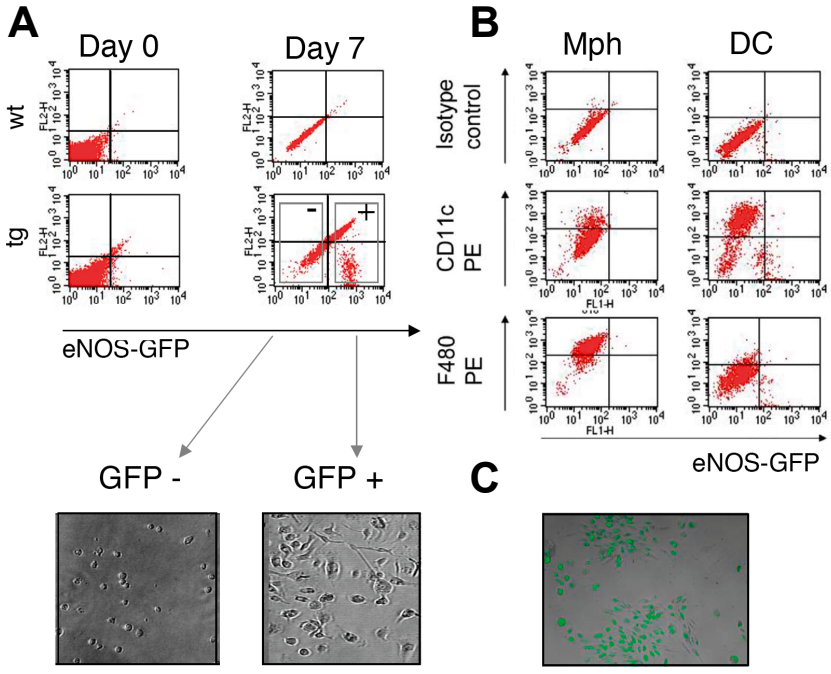

Fig 2. EPC from eNOS-GFP transgenic mice show a specific expression of GFP.

(a) Expression of eNOS was measured in total BM of eNOS-GFP C57Bl/6J transgenic (tg) mice and compared with wildtype (wt) controls at day 0 and in day 7 EPC cultures using flow cytometry and GFP as a fluorescent marker. The FL2 channel shows autofluorescent staining. GFP^+ and GFP^- cells from day 7 EPC cultures were then sorted. Clear spindle shaped morphology typical for EPC was observed in the GFP^+ fraction by phase-contrast microscopy. (b) DC and Mph were cultured for 7 days under their specific growth conditions and measured for eNOS-GFP expression. DC and Mph were stained with antibodies against typical mouse DC (CD11c) and Mph (F4/80) antigens. Only BM cells differentiated under EPC conditions showed a high eNOS-GFP expression, whereas Mph and DC cultures displayed no or hardly any GFP expression in combination with lineage specific antigens. (c) BM cells cultured in an CFU-EC assay, give rise to GFP^+ colonies after 3 days of culturing, demonstrating the specificity of the eNOS marker for the EC lineage.

cells. The GFP$^-$ population hardly re-attached, indicating that these did not represent EPC. To ensure that the GFP reporter specifically tracks EC and EPC, BM cells of the transgenic mice were cultured with either GM-CSF to differentiate them to DC or with M-CSF for Mph$^-$ (fig 2B). In the Mph culture no GFP$^+$ cells were present and over 90% of the culture was F4/80^{hi}. In the DC culture only a very small percentage (3%) of cells was found to express GFP at a low level. However these GFP$^+$ cells did not express CD11c suggesting that these few GFP$^+$ cells were not DC.

To assess the property of BM-derived EPC to differentiate and proliferate in an *in vitro* colony assay and to exclude the possibility of a minute fraction of mature EC growing out in our cultures, we performed an established CFU-EC assay. BM of transgenic mice was plated on fibronectin-coated dishes for 48 hours and non-attaching cells were then replated and assessed for colony outgrowth (GFP$^+$ colonies). There were hardly any cells attached to the plates after two days and these few cells did not survive and/or proliferate in the next 3 days (data not shown). The non-attached fraction however did form GFP$^+$ colonies as shown in figure 2C. This observation was extended when we sorted out the very small GFP$^+$ population, presumably corresponding to a minute fraction of mature EC in BM. When cultured under EPC culture conditions, the GFP$^+$ population did not survive and did

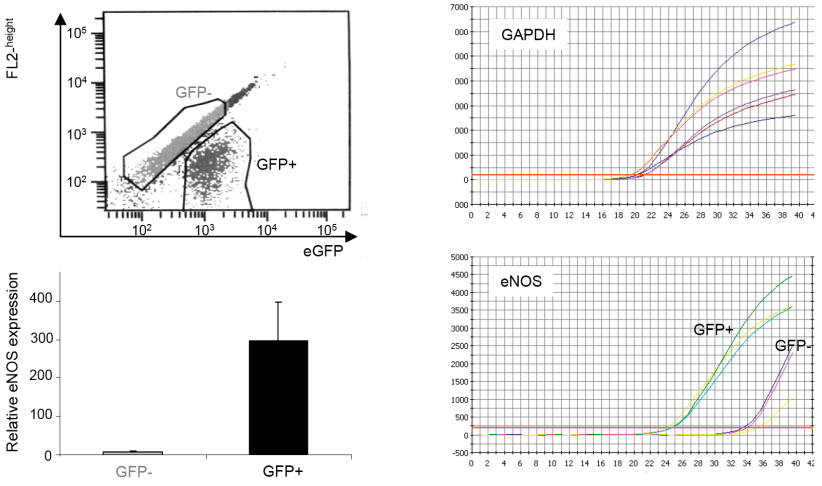

Fig IV. Expression of eNOS mRNA in sorted cell populations.

EPC were cultured for 7 days and GFP$^+$ fraction was separated from the negative cells as shown in upper panel. Total RNA was extracted from the purified populations and eNOS mRNA expression was measured in both fractions relative to normalization genes GAPDH and Actin(not shown) using real time PCR techniques. Relative expression of eNOS normalized to GAPDH was calculated and is shown in lower left panel.

not expand (data not shown), while the GFP$^-$ population proliferated significantly and differentiated into eNOS$^+$ GFP$^+$ cells.

We conclude that using this mouse model EPC differentiation can be tracked allowing identification and separation of true EPC from cells not committed to the endothelial lineage.

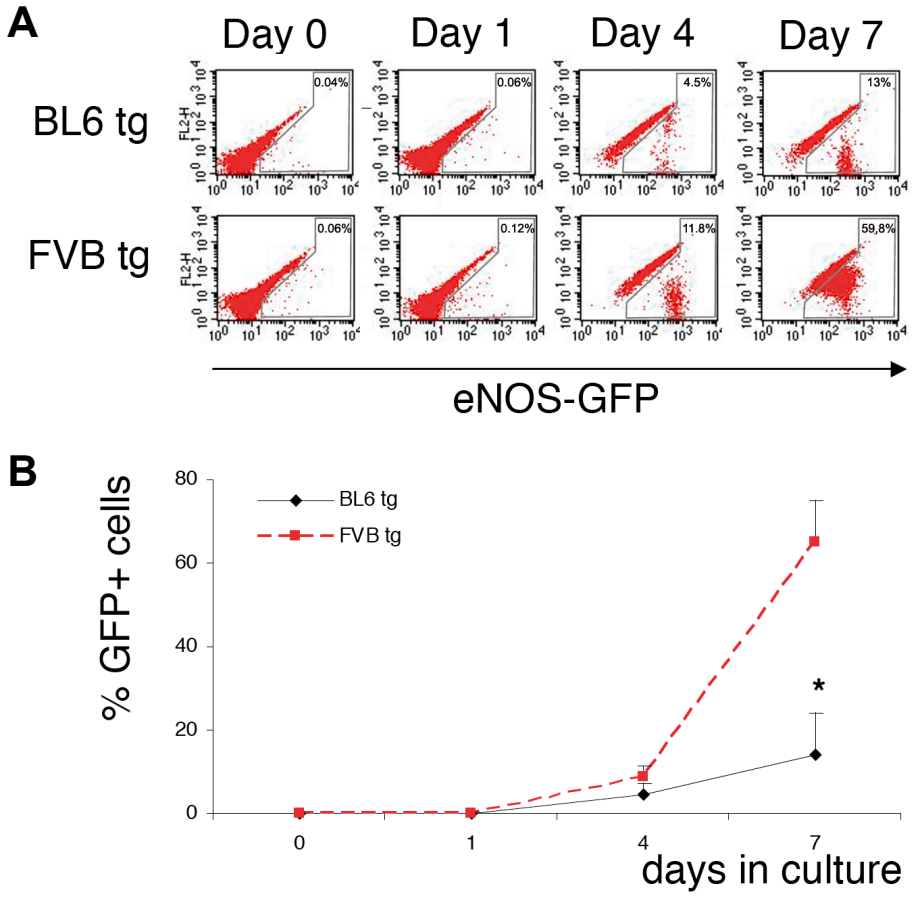

Fig 3. Kinetics of EPC differentiation varies between different mouse strains.

(a) BM of FVB- (FVB tg) and C57BL/6J-transgenic mice (BL6 tg) was cultured to generate EPC and the expression of eNOS-GFP was measured in time by flow cytometry for 7 days. The FL2 channel shows autofluorescence. (b) The percentage of GFP$^+$ cells of the attached cells in culture was measured by flow cytometry and plotted (n=6, for both groups FVB tg and BL6 tg). At day 7, BM from BL6 tg mice showed fewer eNOS-positive EPC than BM from FVB tg, * P< 0.01, BL6 tg versus FVB tg.

EPC differentiation varies between different mouse strains.

To further explore commitment of BM-derived cells towards the endothelial lineage, GFP expression was followed in time up to 7 days. Because there could be differences between mouse strains we studied the kinetics of EPC differentiation in two different genetic backgrounds, C57BL/6J - and FVB eNOS-GFP transgenic mice. At day 0, there was no significant difference in the already very low number of GFP^+. At day 1 the attached cells were GFP^- (Fig.3A), however, at day 4 eNOS-GFP^+ cells appeared in both strains that expanded further in time. At day 4, a trend of higher numbers of GFP^+ cells was observed in the FVB background mice, but this was not statistically significant. At day 7, over four fold more GFP^+ cells were observed for FVB over the C57BL/6J strain (FVB mice 65 ± 11% GFP^+ cells (n=6) vs. C57BL/6J 15 ± 7.5% (n=6), *$P<0.01$) (Fig.3B). These data indicate that eNOS expressing EPC can be derived from BM of both strains tested, but more readily from FVB mice.

Ex vivo expanded EPC from BM are mainly derived from a specific myeloid precursor fraction.

Next we investigated which sub-fraction of the BM contains progenitors for EPC. Based on a two color flow cytometry analysis with ER-MP12 (anti-CD31) and ER-MP20 (anti-Ly-6C), total BM cells can be separated in six phenotypically and functionally distinct subsets[29]. We previously showed that three of these subsets contain myeloid progenitor cells[28,32,33] that can give rise to macrophages and dendritic cells. Here, based on CD31/ Ly-6C profiles, all six subsets were flow-sorted from total BM of both eNOS-GFP transgenic mice and cultured under EPC conditions (Fig 4).

In 4 out of 4 sort experiments GFP^+ EPC appeared in the cultures derived from the $CD31^+$/ $Ly\text{-}6C^+$ (P4) subset. As expected, significantly fewer GFP^+ cells appeared in the culture of the eNOS-GFP C57BL/6J background compared to the eNOS-GFP FVB. We previously demonstrated that almost 80% of this $CD31^+/Ly\text{-}6C^+$ (P4) cell fraction are myeloid progenitor cells indicating that the majority of EPC are derived from these cells. In 2 out of 4 experiments we observed a few GFP^+ cells in the $CD31^{lo}/Ly\text{-}6C^{hi}$ (P6) subset but only in the FVB background. In 1 out of 4 sorting experiments a very small fraction of GFP^+ cells was also seen in the $CD31^{dim/hi}/Ly\text{-}6C^{lo}$ (P1/2) sub-fraction. As this fraction contains lymphoid progenitor cells and hematopoietic stem cells it could be that it takes longer to induce EPC differentiation from this fraction, or that the necessary factors are missing in the *in vitro* culture system used here. In conclusion, the main source of EPC from BM is

the CD31^{+}/Ly-6C^{+} (P4) subset, whereas DC and Mph can also be differentiated from the P1/2 and P6 fraction.

Additional phenotyping of the CD31^{+}/Ly-6C^{+} (P4) subpopulation in comparison to cultured EPC and mature EC (mEC, bEnd3 cells) showed that c-kit was markedly present in the BM

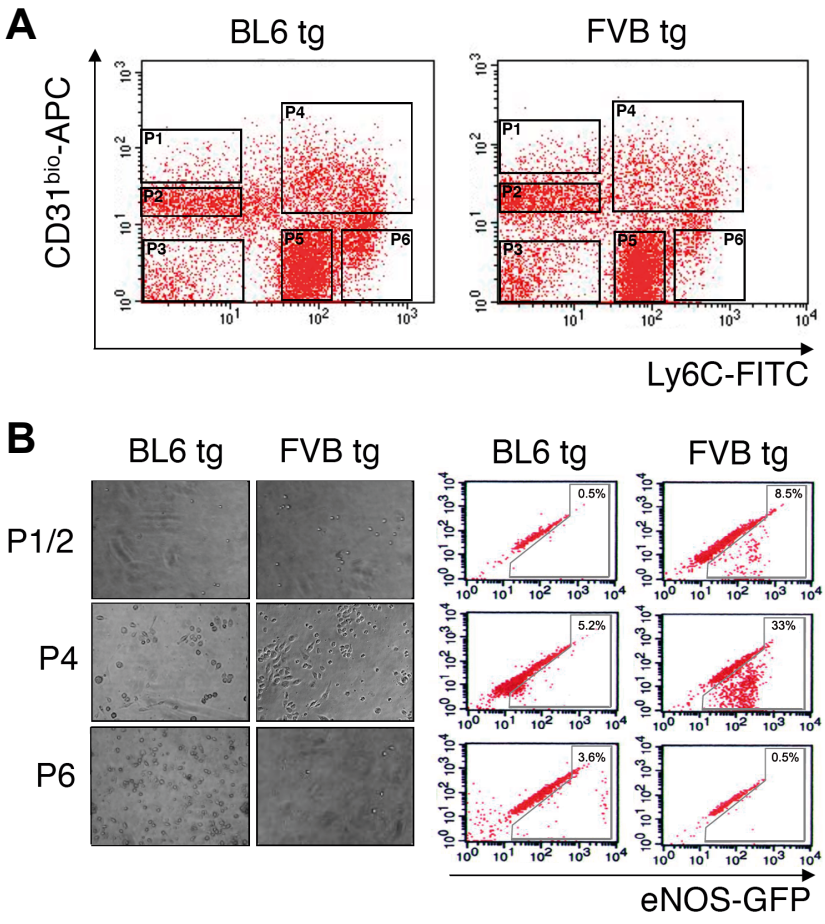

Fig 4. EPC are mainly derived from a specific myeloid CD31^{+} /Ly-6C^{+} precursor fraction of the BM.

(a) Fresh BM (day 0) of C57BL/6J (BL6 tg)- and FVB transgenic mice (FVB tg) was stained with CD31 and Ly-6C antibodies revealing different myeloid fractions of the BM. (b) Several fractions P1/2 (P1 and P2 together), P4 and P6 were sorted by flow cytometry and the fractions were cultured separately. After 7 days the cells were isolated and measured for eNOS-GFP expression by flow cytometry. Cells sorted from the P4 (CD31^{+}/Ly-6C^{+}) subpopulation differentiated most efficiently into EPC. FVB tg mice revealed higher numbers and more efficient EPC differentiation when compared to BL6 tg mice.

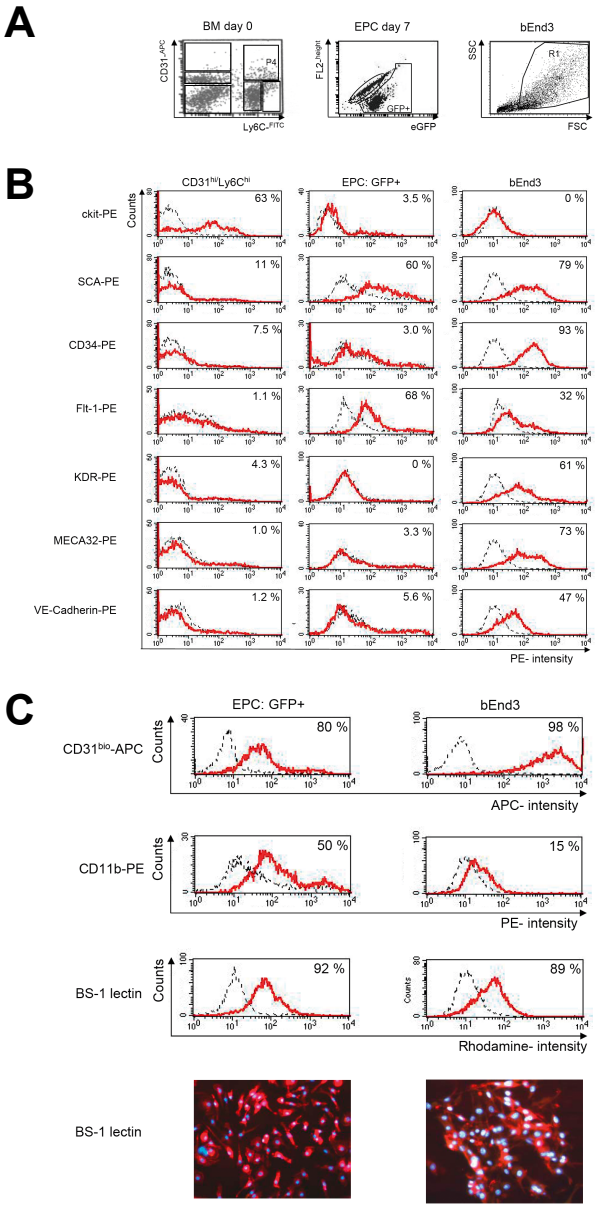

Fig III: Phenotypical characterization of EC differentiation.

A panel of stem cell markers and EC markers was used to characterize three different populations of cells in EC differentiation. These gated cell populations were $CD31^{high}/Ly6C^{high}$ cells in freshly isolated BM, GFP^{+} cells derived from a 7 day EPC culture of eNOS-GFP transgenic FVB mice and as positive EC control we used a murine brain endothelial cell line also known as bEnd3 cells (A).

Harvested cells were stained with a panel of antibodies and representative histograms out of 3 experiments performed are shown (filled line). Isotype controls were used (dotted line) and the percentages of positive cells above this background were calculated (figure 2B).

Panel C shows extra characterization of the GFP^{+} EPC cells compare to mature EC. CD31 expression as well as CD11b expression was analyzed. As expected, an up regulation of CD31 was observed and on the contrary a diminished expression of CD11b, a myeloid marker could be seen. BS-lectin showed strong binding on both cell types and this binding was confirmed with immunohistochemistry, showing a distinct pattern of lectin binding. Similar results were obtained with cultured lung mEC cells derived from lung tissue of eNOS-GFP tg mice, when gated on the eNOS (GFP^{+}) fraction (data not shown). The expression of GFP, reflecting eNOS expression was about 2.5 times higher on mEC compared to EPC. We also observed a marked difference in the side scatter of mEC and EPC (300 vs. 500 respectively, data not shown)

P4 fraction, however very minor in the EPC cultures and absent in the mature EC cultures (Fig III B). A subpopulation of 11% of this P4 fraction showed Sca-1 expression, whereas Sca-1 expression seemed to be highly present on EPC as well as mature EC. The observation that Sca-1 is expressed on EC and even a possible function of expression of Sca-1 on EC has been proposed previously by Luna *et al.*[34]. VEGF-1 (flt-1) is highly expressed on eNOS$^+$ cells, while KDR is not yet detectable. VE-cadherin is positive on a small subset of cells and has been confirmed by immunohistong (data not shown). CD31 is upregulated in EPC fraction and showed an even higher expression on mature EC (Fig IIIC). Myeloid markers such as CD11b were downregulated on GFP$^+$ EPC and even further on mature EC, especially when compared to Mph and DC. BS-1 lectin staining of the total population of both EPC and mEC was confirmed by flowcytometric as well as immunohistochemical analyses. We conclude that, with the notable exception of Sca-1, EPC express higher levels of progenitor/stem cell markers than mature EC and begin to express EC-specific markers while downregulating classical myeloid markers, consistent with a further narrowing of differentiation potential towards the EC lineage.

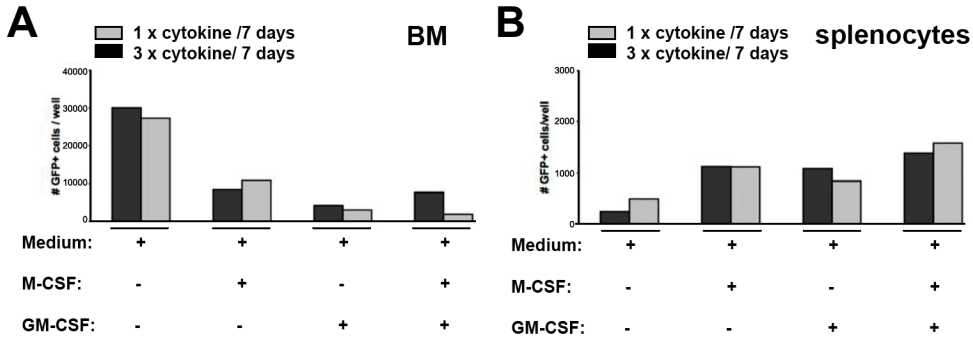

Fig 5. Addition of specific growth factors to EPC cultures has different effects depending on the source of the EPC.

EPC were cultured using either BM (a) or spleen-MNC (b). The cells were cultured under standard optimized EPC conditions (MH$^-$) or with addition of M-CSF (10 ng/ml), GM-CSF (20 ng/ml) or a combination of both. These cytokines were either refreshed every 2 days (black bars) or they were only added once at day 0 (grey-bars). After 7 days cells in culture were counted and the total number of GFP$^+$ cells per well was determined by FACS. A 2-3-fold increase in the number of GFP$^+$ EPC was observed by addition of growth factors in cultures from spleen-derived cells, but not with BM cells. Refreshing the growth factors every two days or addition only once at day 0 showed a similar proliferation/differentiation pattern. A representative experiment is shown.

Spleen derived EPC can be expanded using myeloid specific growth factors

The therapeutic potential of EPC has elicited a number of studies that demonstrated that myeloid growth factors can stimulate recruitment, differentiation or outgrowth of EPC and may have favorable effects on their function[35-37]. Therefore, the effects of GM-CSF and M-CSF on EPC differentiation from BM were determined. Addition of these myeloid growth factors to the cultures lowered the numbers of EPC (GFP^+ cells) derived from the BM precursors (Fig 5A). Other sources than BM have been used to derive human EPC and murine EPC. Human EPC can be cultured from $CD14^+$ mononuclear cell fractions isolated from peripheral blood mononuclear cells (PB-MNC)[10,11] or from $CD34^+$ progenitor cells isolated from G-CSF-mobilized peripheral blood (PB) stem cells[37] umbilical cord blood[13] or BM[38]. Murine EPC have been cultured from BM and spleen. The mononuclear cell fraction of the spleen is often used as a homologue of PB-MNC from mice as it is described as a reservoir of peripheral blood stem/progenitor cells[39]. Spleen-derived murine EPC have similar functional (angiogenic) and phenotypic characteristics as BM-derived EPC (data not shown), but they show a lower proliferation capacity. Using the same culture conditions as described above for the generation of BM-derived EPC, spleen-derived cultures yielded 10-50 fold lower numbers of GFP^+ EPC (Fig 5B). Addition of myeloid growth factors to spleen-derived cultures showed an increase in the number of EPC. Thus addition of myeloid growth factors as GM-CSF and M-CSF could be useful for expanding PB-or spleen derived EPC *ex vivo*, but not for BM derived EPC.

Discussion

In this study we characterized the *ex vivo* commitment of BM-precursors towards endothelial cells in terms of phenotype, lineage potential, differentiation from BM precursors and angiogenic properties. In order to address these issues in detail and to have an endothelium specific marker we made use of eNOS-GFP transgenic mice. This well characterized system[27] allows a careful appreciation of the relationship between myeloid and endothelial lineages. Our report emphasizes the high phenotypic overlap and close relationship of EPC, DC and Mph. Consequently, frequently used markers for EPC, such as the uptake of acLDL and binding of Ulex, are relatively unspecific as these are also markers for Mph. Despite this high phenotypic overlap of EPC, DC and Mph, the capacity of EPC

to support angiogenesis is a unique feature of EPC when compared to DC and Mph. While we could demonstrate a potent angiogenic capacity in the CM of EPC, we observed that only a small fraction of the EPC did incorporate (as do mature EC) in tubes. The majority of the EPC appears to function as pericytes and localize around the tubes and under the junctions, but do not form an integral part of it. Other investigators also found that attaching cells derived from BM or PBMC under culture conditions with VEGF did not differentiate into EC, but stimulated angiogenesis in other ways[11,40]. Therefore, the term endothelial progenitor cells might not be an adequate definition of the total cell culture, as not all cells might become true endothelial cells under the conditions used. Although we generally refer to these attaching cells with angiogenic capacity as EPC, following the consensus in the field, the term angiogenic myeloid cells may be more appropriate. Nevertheless, the cells referred to as EPC are different from mature EC, as demonstrated in the CFU-EC assays and by phenotypic analysis. EPC also express higher levels of stem cell markers, but lower levels of eNOS, although they are clearly positive for this marker.

We showed that there is a strain difference between FVB and C57Bl/6J mice in their capacity to generate EPC from BM precursors. FVB mice are less susceptible for atherosclerosis[41] and this might possibly indicate a role for the plasticity of bone-marrow precursors to differentiate towards EC. As a corollary, we conclude that C57Bl/6J mice might not be the best strain to choose for studying short-term cultured murine EPC.

A number of studies indicated that myeloid growth factors like GM-CSF can be used to augment neovascularization in animal models and in patients[42]. In this study, only for spleen-derived EPC the number of eNOS-GFP$^+$ EPC increased. In BM, addition of M-CSF and GM-CSF to the culture resulted in a decreased number of EPC, probably due to extensive expansion of myeloid progenitors that are driven into another differentiation lineage than EC, such as DC and Mph.

It is becoming increasingly apparent that cells of the myeloid lineage display a high plasticity and that some of these seemingly “lineage-committed” myeloid cells can, under specific growth conditions, differentiate into cells of another lineage with distinct functional properties[20,25,43]. For instance, in the presence of inflammatory cytokines, the normal differentiation of monocytes into macrophages can be skewed to yield dendritic cells[26]. Another example is the differentiation of myeloid cells into cells of the mesenchymal lineages[44]. Likewise, several reports have described the myeloid character of endothelial cells[12,20,45]. Cultures of adhered mononuclear cells[12] or dendritic cells[46,47] grown under stringent angiogenic differentiation conditions have been shown to differentiate into

endothelial like cells. We argue that this large degree of plasticity among cells of the myeloid lineage and the close phenotypic overlap between many of these different myeloid lineages (including cells that stimulate angiogenesis) cautions the use of these cells in clinical cell transplantation protocols aimed to augment neovascularization in peripheral or cardiac ischemia. In particular when early outgrowth EPC are derived from patients that are subject to chronic systemic inflammation, transplanted cells might have sub-optimal angiogenic properties or even induce an unwanted immunological response.

In the present study, we observed that LPS-stimulated EPC cultures have the capacity, although to a low extent, to induce T-cell proliferation in an MLR.

Using short cultured cell sorting experiments, we here show that the best and almost exclusive source for murine EPC are the myeloid progenitors in the BM. This myeloid character of EPC is in line with a recent study from Dimmeler and co-workers showing that $CD34^{low}CD14^{+}$ cells in peripheral blood are a major source of EPC[10]. Translating our results to the human situation suggest that further purification of human $CD34^{+}$ cells to include only $CD33^{+}$ (immature myeloid marker) /$CD34^{+}$ myeloid progenitors, but exclude contaminating cells that may yield unwanted side-effects, could be of clinical relevance. Further experiments have to determine whether human myeloid progenitor cells from BM or cord blood provide a superior source of EPC.

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Chapter 6

Endothelial Progenitor Cell Dysfunction in Hyperglycemia originates in Myeloid Precursor Cells in the Bone Marrow

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Abstract

Bone marrow (BM)-derived endothelial progenitor cells (EPC) contribute to endothelial maintenance and repair. Risk factors for ischemic vascular disease such as diabetes mellitus not only affect the mature endothelium, but also lead to EPC dysfunction. To explore the impact of hyperglycemia on the differentiation of EPC from bone marrow progenitor cells, mice were maintained hyperglycemic for 6 weeks. Cultures generating EPC, macrophages (Mph) or dendritic cells (DC) from hyperglycemic BM yielded 40% fewer EPC and 50% more Mph compared to control bone marrow. These changes were directly related to the HbA1C levels of the donor mice. In contrast, BM-derived DC numbers were not affected by hyperglycemia. The composition of the BM was not altered; in particular the numbers of $CD31^+/Ly6C^+$ cells, which serve as common progenitors for EPC, Mph and DC were unaffected. In addition to their lower quantity, BM-derived EPC from hyperglycemic mice were less angiogenic and more pro-inflammatory in terms of endocytosis, T-cell activation and IL-12 production. HMGCoA reductase inhibition by statin supplementation of the culture medium counteracted these hyperglycemia-induced changes. Our data indicate that EPC dysfunction in diabetes is BM-derived, due to hyperglycemia-induced alteration of myeloid progenitor cell differentiation.

Introduction

Loss of endothelial integrity and an impaired capacity for ischemia-induced neovascularization leads to ischemic vascular disease in diabetes[1-3]. A recently identified risk factor for these vascular complications is dysfunction of bone marrow (BM)-derived endothelial progenitor cells (EPC). Under normal circumstances, EPC contribute to vascular homeostasis by replacing apoptotic or lost endothelial cells[4,5]. In addition, EPC can home to sites of denudation[6], ischemia or elevated shear stress to stimulate neovascularisation or arteriogenesis[7,8]. In patients with type 1[9] and 2 diabetes[10] and in diabetic animals[11-13], the number of circulating EPC is decreased and functional parameters such as adhesion, migration and the paracrine secretion of proangiogenic factors are impaired, likely as a consequence of hyperglycemia. Moreover, a recent study showed that the number of circulating EPC inversely correlates with the severity of peripheral vascular complications of patients with type 2 diabetes further supporting a role for EPC dysfunction in the pathogenesis of ischemic vascular disease[14]. Following these notions, EPC are increasingly recognized as a potential therapeutic target for the prevention of ischemic vascular disease. However, the molecular mechanisms underlying EPC dysfunction in diabetes are complex and may include reduced cell survival[11,15] due to an increased sensitivity to oxidative stress[16] or activation of a p53-dependent pathway that leads to cellular senescence[16,17].

Human EPC can be derived from either $CD34^+$, $CD34^-$ or $CD34^{low}$ cells and can be cultured from BM aspirates and peripheral blood $CD14^+$ mononuclear cell fractions[18-21]. This heterogeneity in precursor cells likely reflects the different stages of EPC as they mature from the early bone marrow-derived stem cells towards mature vascular endothelial cells.

Many studies have shown that cells of the myeloid lineage display high plasticity and that seemingly "lineage-committed" myeloid cells, given the appropriate conditions, can differentiate into cells of another lineage with different functional properties[22]. For example, in the presence of inflammatory cytokines, the normal differentiation of monocytes into macrophages (Mph) can be skewed to yield dendritic cells (DC)[23]. We recently demonstrated that angiogenic EPC develop from an immature, $CD31^+$/Ly-$6C^+$ myeloid progenitor fraction in mouse BM[24]. Upon stimulation by the proper differentiation factors, Mph and DC are also derived from this fraction[25,26], suggesting a common myeloid progenitor pool for EPC, Mph, granulocytes, monocytes and DC.

As diabetes is increasingly appreciated as a systemic pro-inflammatory state[27,28] that might affect cell-fate specification or developmental decisions, we here investigated the differentiation of EPC, Mph and DC from BM-derived myeloid progenitor cells of hyperglycemic and normoglycemic mice. Our results support the hypothesis that EPC dysfunction in diabetes is bone marrow-derived due to hyperglycemia-induced alterations in differentiation of myeloid progenitor cells.

Materials and methods

Animals

Moderate to severe hyperglycemia was induced in 6 week old C57BL/6J and FVB/N mice (Harlan, Horst, The Netherlands) by intraperitoneal (IP) injections of streptozotocin (STZ, 80 mg/kg body weight; Sigma-Aldrich) in 0.05 mol/l Na-Citrate buffer pH 4.5, on two consecutive days. Blood glucose levels were examined by glucose-oxidase technique (OneTouch Ultra system, Lifescan) 1 week after STZ injection and mice with blood glucose levels below 20 mmol/l mice received two additional injections on two consecutive days. When necessary this procedure was repeated a third time. STZ treatment was stopped when blood glucose levels above 20 mmol/l were reached and glucose levels and total body weight were monitored weekly for a period of 6 weeks. Control mice were injected with buffer only at the onset of the experiments. Mice from the STZ treatment groups that showed no elevated blood glucose levels (below 15 mmol/l) were excluded from the study. All procedures involving animal handling were in accordance to national and institutional guidelines of the Erasmus MC, Rotterdam, The Netherlands.

Glycosylated hemoglobin

Glycosylated hemoglobin (HbA1C) levels were measured by High Performance Liquid Chromatography (HPLC) using a reverse-phase cation exchange column and detected by a dual wavelength colorimetric (415 and 500 nm) analyzer (ADAMS A1c HA8160; Arkray).

Isolation and differentiation of murine EPC, DC and Mph

BM-cell suspensions were prepared by flushing femora and tibiae with RPMI medium (Gibco) supplemented with 10% FCS (Gibco) and antibiotics (penicillin/streptomycin, Gibco). To culture EPC, total BM cell suspensions were plated at a density of 1.25 x 10^6

cells per cm^2 in 24-well plates (Nunc) coated with fibronectin (10 µg/ml, Sigma). Cells were cultured for 7 days in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV).

For Mph and DC, 2 $x10^6$ BM cells were cultured in complete RPMI medium described above and plated on 10 cm Petri dishes (BD Biosciences). Medium was replaced every two days. For DC and Mph cultures, the medium was supplemented with 20 ng/ml recombinant murine GM-CSF (Biosource) and 10 ng/ml recombinant murine M-CSF (Peprotech), respectively. Atorvastatin (Pfizer) was dissolved in ethanol and, in indicated experiments, added to the medium to a final concentration of 0.1 µmol/l. Medium used in the control experiments contained ethanol at a similar final concentration ($5x10^{-5}$ %) as present in the cultures treated with atorvastatin.

Monoclonal antibodies, conjugates and flow-cytrometric analysis

Undiluted culture supernatant of the hybridoma F4/80 was directly used for staining. ER-MP12/CD31 was purified and biotinylated and ER-MP20/Ly-6C was conjugated to FITC[25,29]. Phycoerythrin-(PE-) labeled CD11c and biotinylated anti-MHC class II antibodies were purchased from BD Biosciences. Secondary FITC- or R-PE-labeled goat anti-rat IgG antibodies (mouse-absorbed; GαR-FITC or GαR-PE) were purchased from Caltag Laboratories. Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Biosciences). For EPC characterization, cells were stained with rhodamine-labeled Bandeiraea simplicifolia lectin (10µg/ml, BSL-1, Vector Labs) for 1 h. To measure the uptake of Dil-labeled acetylated LDL (acLDL, Molecular Probes), cells were incubated with acLDL (2.4 µg/ml) at 37°C for 2h. Flowcytometric analyses were done on a Calibur flowcytometer (BD Biosciences) using CellQuest software (BD Biosciences).

Cytokine detection

For cytokine measurements EPC were incubated with 50 ng/ml lipopolysaccharide (LPS, Sigma) overnight at 37°C. Culture supernatants were collected and cells were counted for normalization. IL-12p40 and IL-12p70 ELISA kits (R&D) were used according to the manufacturer's protocol.

MLR assay

Mixed leukocyte reactions (MLR) were done with allogeneic T cells from C57BL/6J or FVB/N splenocytes. Cells were incubated with antibodies recognizing CD11b, CD45 and MHCII and anti-rat IgG microbeads. Naive T cells were obtained by negative selection using a magnetic cell sorter. DC, Mph and EPC were irradiated sub-lethally. T cells (1.5 x 10^5 cells/well) were added to varying concentrations of stimulator cells depending on the desired stimulator-responder cell ratio. Proliferation of T cells was measured after 4 days by uptake of ^{3}H-thymidine (1 μCi/well, DuPont-NEN) and measured as counts per minute (cpm).

In vitro angiogenesis assay.

Conditioned media were obtained by replacing the medium of 6-day EPC cultures with serum-free EC basal medium-2 (Clonetics) supplemented with EGM-2 single aliquots (without vascular endothelial growth factor and basic fibroblast growth factor) and culturing the cells for an additional 20 h. EPC were counted and conditioned media were diluted to correct for cell numbers. After 14 h, tube formation by HUVECs was measured by staining the viable cells with Calcein-AM (5 μg/ml, Molecular Probes). HUVEC cultures were isolated as described[30] and used at passage 3 or less. For quantification, total tube area was determined using images obtained with an inverted fluorescence microscope and Scion Imaging software (Scion Corporation) and expressed in arbitrary units of tube length.

Endocytosis assay

Uptake of dextran-FITC was determined for 30 min, both at 4°C and 37°C. Subsequently, the cells were washed three times with PBS/BSA1% and total uptake of FITC label was measured by FACS analyses and expressed by the difference in geometric mean that resulted from subtracting the values obtained at 4°C from the values obtained at 37°C.

Statistical analysis

Differences between treatment groups were analyzed Student's *t* test. Results are expressed as mean ± SD. Linear regression analyses and Pearson correlation were used for comparison of the number of EPC/Mph and HbA1c. Probability values of $P < 0.05$ were considered statistically significant.

Results

Hyperglycemia induction in mice

In both mouse strains used (C57BL/6J and FVB/N), STZ treatment increased blood glucose levels up to 3.5 fold, associated with increased HbA1C levels (Table 1). Body weight was 25-30% reduced in hyperglycemic mice of both strains when compared to normoglycemic control animals. Total BM cell isolations from tibiae and femora yielded an equal number of cells in hyperglycemic and control C57BL/6J mice whereas in FVB/N mice the hyperglycemic state was associated with a minor 1.3 fold increase in the number of BM-derived cells.

	C57BL/6J Control	C57BL/6J STZ	FVB/N Control	FVB/N STZ
n	18	26	17	23
Blood glucose (mmol/l)	6.7 ± 2.0	24.1 ± 5.5 *	9.1 ± 1.8	27.8 ± 4.5 *
HbA1c %	3.9 ± 0.2	7.6 ± 0.7 *	4.1 ± 0.5	5.9 ± 0.5 *
Weight (gram)	28.5 ± 2.2	20.0 ± 1.2 *	30.5 ± 2.3	22.5 ± 2.9 *
Bone marrow cells $x10^8$	0.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.3

Table 1. Mouse characteristics.

Parameters were measured after 6 weeks of STZ-induced hyperglycemia or in littermate control mice matched for age and gender that were injected with buffer. Values represent mean ± SD, *: $P < 0.05$ v.s. buffer.

Hyperglycemia differentially alters the potential of BM cells to yield EPC, Mph and DC

To determine the effects of hyperglycemia on the potential of BM progenitors to generate EPC, total BM cells were harvested from hyperglycemic and control mice and equal numbers of cells were cultured for 7 days to differentiate into EPC. We previously provided evidence that EPC, Mph and DC may share a common myeloid progenitor pool in the BM[24]. Therefore, the potential of the BM cells to differentiate into $F480^+$ Mph and $CD11c^+$ DC, in M-CSF- and GM-CSF-stimulated cultures, respectively, was assessed in parallel to the EPC cultures. In EPC cultures from BM of hyperglycemic mice from both strains we observed on average a 40% reduction in the number of EPC, identified as cells positive for

CD31 and acLDL (Fig. 1A). In contrast, the number of Mph was approximately 50% increased. No differences were observed in the yield of DC in cultures from both strains of mice. Figure 1B shows that the number of EPC obtained inversely correlated with peripheral blood HbA1c levels of individual mice, indicating that the observed decrease in EPC numbers directly relates to the degree of hyperglycemia. In contrast, the number of Mph showed a positive correlation with the degree of hyperglycemia whereas the varying numbers of DC derived from the cultures showed no relationship with HbA1c levels. Taken

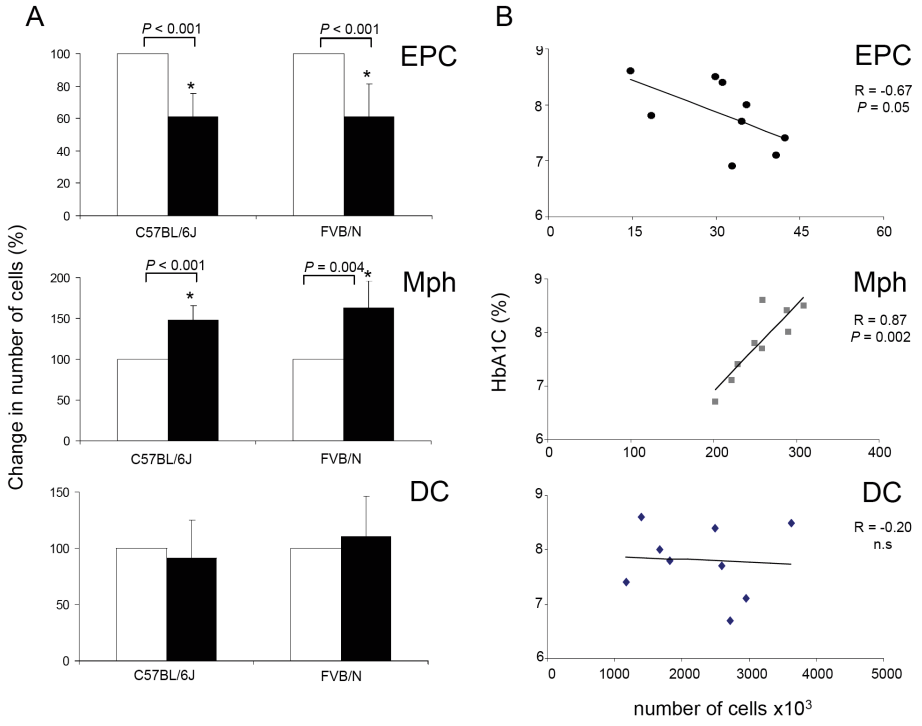
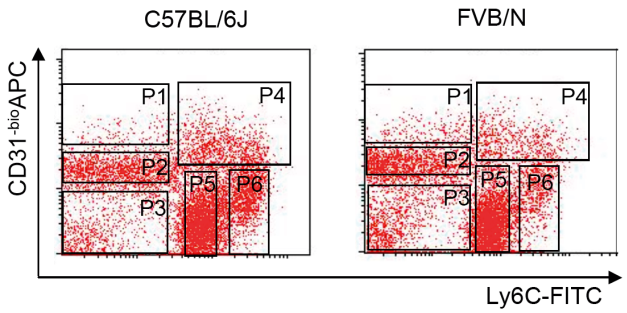


Figure 1. Hyperglycemia alters the differentiation potential of myeloid progenitors in BM.
(A) Relative change in the number of bone marrow-derived EPC, Mph and DC from STZ-treated mice (black bars) compared to control mice (white bars). Values for EPC, Mph and DC derived from BM from mice treated with buffer are set to 100%. Top panel: number of $CD31^{high}$ /Dil-acLDL-positive, attaching cells in EPC cultures (C57BL/6J; 4 experiments, n_{buffer} = 18 and n_{STZ} = 26 and FVB/N; 5 experiments, n_{buffer}= 17 and n_{STZ} = 23). Middle panel: the number of F4/80 positive cells in M-CSF-stimulated Mph cultures (C57BL/6J; 3 experiments, n_{buffer} = 13 and n_{STZ} = 18 and FVB/N; 3 experiments, n_{buffer} = 13 and n_{STZ} = 16). Bottom panel: number of CD11c positive cells in GM-CSF-stimulated dendritic cell cultures (C57BL/6J; 3 experiments, n_{buffer} = 13 and n_{STZ} = 18 and FVB/N; 3 experiments, n_{buffer} = 13 and n_{STZ} = 16). (B) Correlations between the number of cultured EPC (top panel), Mph (middle panel) and DC (bottom panel) and hyperglycemia as assessed by HbA1c. Representative experiment using BM from hyperglycemic C57BL/6J mice (n = 9).

together, hyperglycemia affects myeloid progenitor cells in BM and alters their potential to differentiate into different myeloid lineages.

Hyperglycemia has no major quantitative impact on the subpopulation composition of myeloid progenitors of the BM

To evaluate if the observed altered number of bone marrow-derived EPC and Mph from hyperglycemic mice was due to quantitative shifts in myeloid progenitor fractions of the BM, total BM was immunostained using ER-MP12 (anti-mouse CD31) and ER-MP20 (anti-mouse Ly-6C) and analyzed by flowcytometry. Using this combination of markers[25], 6 distinct subpopulations of BM cells can be identified, each with varying degrees of lineage commitment and progenitor potential (Figure 2). The populations consist of: (P1) 70% blast



	C57BL/6J Change %	FVB/N Change %
$CD31^{high}/Ly6C^{low}$ (P1)	112 ± 34	104 ± 62
$CD31^{med}/Ly6C^{low}$ (P2)	58 ± 10*	57 ± 30*
$CD31^{low}/Ly6C^{low}$ (P3)	108 ± 18	104 ± 16
$CD31^{high}/Ly6C^{high}$ (P4)	128 ± 54	121 ± 56
$CD31^{low}/Ly6C^{med}$ (P5)	148 ± 63	104 ± 35
$CD31^{low}/Ly6C^{high}$ (P6)	112 ± 95	129 ± 98

Figure 2. Hyperglycemia has no major quantitative impact on the myeloid progenitor fractions of the BM.

BM of the control and hyperglycemic mice was harvested and immediately stained with anti-CD31 and anti-Ly-6C. Using this combination of markers, 6 distinct populations can be identified (P1-P6). For each subpopulation, the average increase or decrease in BM cell fractions from hyperglycemic mice is depicted as the percentage change relative to nondiabetic mice. In the top panel representative scatter plots from hyperglycemic BM are shown for each strain. For both strains the average percentage and standard deviations were calculated from 3 experiments (C57BL/6J: $n_{buffer} = 15$ and $n_{STZ} = 18$ and FVB/N; $n_{buffer} = 13$ and $n_{STZ} = 14$, $*P < 0.001$)

cells and 25% lymphoid cells; (P2) lymphoid cells; (P3) erythroid cells; (P4) myeloid progenitors and plasmacytoid cells; (P5) granulocytes; (P6) 75% monocytes and 20% monocyte progenitors[25]. As previously shown, Mph and DC can originate from $CD31^{high}$/$Ly6C^{low}$(P1), $CD31^{high}$/$Ly6C^{high}$(P4) and $CD31^{low}$/$Ly6C^{high}$(P6) fractions[25,26]. However, short term cultured EPC are mainly derived from the $CD31^{high}$/$Ly6C^{high}$ fraction[24]. When comparing the BM myeloid cell subpopulations from hyperglycemic and control mice, we found no significant quantitative differences in the three fractions from which EPC, Mph and DC can be derived in short term cultures (Figure 2). The only subpopulation that was altered was the $CD31^{med}$/$Ly6C^{low}$ lymphoid and HSC fraction (P2) that was 40% decreased in the BM of hyperglycemic mice of both strains. In line with the observation that the total cell number that can be harvested from the BM of these mice is not, or only marginally changed (Table 1), we conclude that hyperglycemia does not affect the quantitative composition of the myeloid progenitor subpopulations in the BM, but rather alters their differentiation potential.

BM-derived EPC from hyperglycemic mice display a pro-inflammatory phenotype

To investigate whether functional properties of EPC cultured from hyperglycemic BM are altered in comparison to those from control BM, we compared their angiogenic and pro-inflammatory potentials (Table 2). We first determined the angiogenic properties of EPC-conditioned medium in an *in vitro* angiogenesis model. When HUVEC were seeded on matrigel and maintained for 14 h in conditioned medium of EPC derived from control C57BL/6J and FVB/N mice, tube formation was markedly stimulated when compared to non-conditioned medium (non-conditioned media 0.37 ± 0.13 A.U. of tube formation (n = 3) $P = 0.04$ and $P < 0.001$ respectively). Conditioned media of EPC from FVB/N mice induced more capillary formation than EPC-conditioned media of C57BL/6J mice ($P = 0.01$). In contrast, conditioned media of EPC from hyperglycemic mice supported tube formation by only 43% (C57BL/6J, $P = 0.03$) and 35 % (FVB/N, $P = 0.02$) compared to the control values (Table 2). Next we examined to what extent the different EPC display pro-inflammatory properties that are typical for myeloid antigen-presenting cells like the ability to endocytose, the capacity to activate T cell proliferation and the expression of pro-inflammatory cytokines. Table 2 shows that EPC from hyperglycemic mice displayed a trend towards a higher capacity to endocytose large dextran molecules, when compared to EPC from normoglycemic mice. C57BL/6J EPC show a higher capacity to endocytose when compared to FVB/N mice ($P < 0.001$). To assess whether the capacity of Mph derived

	C57BL/6J Control (n)	C57BL/6J STZ (n)	*P* value	FVB/N Control (n)	FVB/N STZ (n)	*P* value
Angiogenesis length tubes (A.U.)	0.47 ± 0.17 (8)	0.27 ± 0.12 (8)	0.03 *	0.77 ± 0.19 (12)	0.50 ± 0.20 (12)	0.02*
Endocytosis EPC Δ geo mean	63.6 ± 18.0 (9)	78.8 ± 21.2 (15)	0.12	20.7± 17.9 (8)	42.3 ± 15.2 (8)	0.09
Endocytosis Mph Δ geo mean	47.0 ± 8.6 (3)	62.7 ± 8.0 (3)	0.13	78.3 ± 7.6 (3)	68.8 ± 11.4 (3)	0.28
MLR ^{3}H-thymidine incorporation (%)	100 (9)	176 ± 71 (15)	0.15	100 (6)	139 ± 27 (8)	0.08
IL-12p40 concentration (pg/ml)	26.9 ± 13.2 (8)	64.4 ± 19.3 (8)	0.001*	29.3 ± 13.5 (8)	56.6 ± 24.3 (8)	0.02*
IL-12p70 concentration (pg/ml)	78.4 ± 28.1 (8)	155 ± 52.5 (8)	0.006*	62.7 ± 44.3 (8)	262 ± 63.1 (8)	< 0.001*

Table 2. Angiogenic and pro-inflammatory properties.
Functional analyses of EPC and Mph cultured for 7 days from control and hyperglycemic BM. Listed are the capacity of EPC-conditioned media to stimulate *in vitro* formation of tubular structures by HUVEC (n = 8, each group), the capacity of EPC and Mph to endocytose large dextran molecules labeled with FITC as measured by FACS analyses and expressed as the delta geo mean (Δ), the capacity of EPC to stimulate T cell proliferation as assessed by allogeneic MLR (determined by measuring ^{3}H-thymidine incorporation and expressed in percentage compared to EPC from controls), and the expression of the pro-inflammatory cytokine IL-12p40 and IL-12 p70 production by EPC after LPS stimulation (n = 8 per group).

from hyperglycemic mice to endocytose was altered, Mph were cultured from hyperglycemic and control mice. Mph derived from hyperglycemic BM from C57BL6/J mice showed a trend towards a higher capacity to take up large dextran molecules, while FVB/N-derived Mph showed a small but not significant decrease when derived from hyperglycemic BM.

When the EPC fractions were tested for their capacity to stimulate T-cell proliferation in mixed lymphocyte reaction assays (MLR), again for both strains we observed a trend towards a higher inflammatory capacity of the EPC from hyperglycemic BM. This was supported by our findings on the ability of the cells to produce IL-12 upon LPS stimulation IL-12p70, the active form of the pro-inflammatory IL-12, can regulate T cell-mediated immune responses by promoting Th1 development and we have previously shown that it is the predominant IL-12 subtype produced by EPC[24]. As shown in table 2, IL-12p70 levels were elevated in the conditioned medium of EPC from hyperglycemic mice, ranging from 2.3 fold for C57BL/6J mice (P = 0.006) up to 4 fold for FVB/N mice (P < 0.001). The IL-12p40 subunit was also about 2 fold higher in supernatants from EPC derived from hyperglycemic mice as compared to the supernatants of control EPC (C57BL/6J mice (P = 0.001), FVB/N mice (P = 0.02). Because elevated inflammatory properties were observed under hyperglycemic conditions, BM-derived EPC were further phenotypically

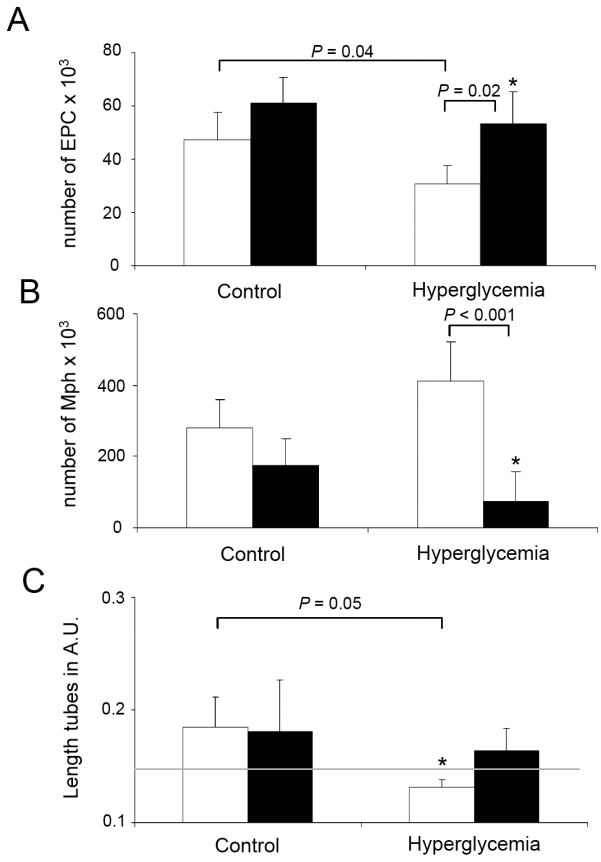


Figure 3. Atorvastatin reverses hyperglycemia-induced changes in progenitor commitment and EPC function.

Number of EPC (A) and Mph (B) cultured from BM of control and hyperglycemic mice (n = 6 per group). (C) Tube formation in arbitrary units (AU) in response to medium conditioned by EPC from control and hyperglycemic mice (n = 6 per group) cultured in the absence (white bars) or presence of atorvastatine (black bars).

characterized by flow cytometry analyses. However, when EPC from hyperglycemic and control mice were stained with either anti-F480 or anti-CD11c antibodies, to reveal a more Mph- or DC-like phenotype, no significant differences could be observed (data not shown). Taken together, EPC derived from hyperglycemic mice have a reduced angiogenic capacity but are more pro-inflammatory as they secrete more IL-12 and they show a trend for an elevated capability to endocytose exogenous material and to stimulate naïve T cells.

Statin treatment in vitro restores affected progenitor commitment and EPC function of hyperglycemic mice

HMG-CoA reductase inhibitors have been shown to be potent anti-inflammatory agents[31]. Moreover, they elevate EPC numbers *in vitro*[32] and *in vivo*[33] and interfere with the differentiation of monocytes towards Mph[34,35]. Therefore, in separate experiments, we explored the effect of atorvastatin on the hyperglycemia-induced effects on BM-derived EPC and Mph. BM cells of hyper- and normoglycemic C57BL/6J mice were cultured for 7 days to obtain EPC and Mph. As expected, lower numbers of EPC were derived from BM of hyperglycemic mice ($P = 0.04$). When atorvastatin (0.1 μmol/l) was present in EPC cultures from hyperglycemic mouse BM, the number of EPC was normalized to levels obtained from control mice (Figure 3A). Conversely, the number of Mph generated in M-CSF stimulated cultures of both hyperglycemic and control BM was markedly reduced in the presence of atorvastatin ($P < 0.001$; Figure 3B).

To evaluate the effect of atorvastatin on EPC function, an *in vitro* angiogenesis assay was performed with conditioned medium of EPC cultured in the presence of atorvastatin. As shown in figure 3C, we again observed significant reduction in tube formation when HUVEC were subjected to conditioned media of EPC derived from hyperglycemic mice ($P = 0.05$). This loss in paracrine angiogenic capacity was attenuated when the EPC from hyperglycemic mice were cultured in the presence of atorvastatin.

Discussion

Diabetes can be envisioned as a disease characterized by a chronic systemic inflammatory state disturbing the function of multiple vital body systems, in particular the vasculature[27,28]. We hypothesized that hampered differentiation of EPC from myeloid progenitor cells in the BM might contribute significantly to the suboptimal endothelial repair under hyperglycemic conditions. Indeed, we observed a marked 40% reduction in the number of EPC that could be cultured from BM of mice that were made hyperglycemic by streptozotocin treatment. Similar to what we previously observed for the number of EPC that could be cultured from PB-MNC from patients with type 1 diabetes[9], the observed reduction was inversely correlated with HbA1C levels suggesting a causal role for hyperglycemia. In addition, EPC from hyperglycemic murine BM displayed impaired angiogenic activity. Again, EPC from patients with type 1 diabetes are likewise impaired in

angiogenicity[9]. Our findings confirm that EPC-mediated neovascularization may be impaired in diabetes due to a reduction in number and angiogenic capacity of the circulating EPC, caused by their defective development from myeloid BM progenitors. Indeed, others have shown that when progenitor cells derived from a hyperglycemic background are transplanted into mice they fail to augment ischemia-induced neovascularization[12,13] and have decreased capacity for re-endothelialization following arterial injury[36]. However, our results also demonstrate that EPC derived from hyperglycemic BM display a pro-inflammatory phenotype as their capacities to endocytose, to activate T-cells and to produce IL-12 are increased. By mRNA expression profiling we recently demonstrated that human EPC from patients with type 1 diabetes over express numerous pro-inflammatory genes known to be associated with hyperglycemia and oxidative stress, including osteopontin, plasminogen activator type 1, lectin-like oxidized LDL receptor, thrombomodulin and type IV collagen[37]. Together these data suggest that when EPC from a diabetic background home to sites of ischemia or vascular injury, the pro-inflammatory nature of these cells may contribute to an adverse (immune) response that may be pro-atherogenic or contribute to the formation of neointima. EPC dysfunction was previously proposed to explain the pro-atherogenic nature of transplanted BM-MNC from apolipoprotein E-knockout mice[38] and may limit the direct use of autologous progenitor cell transplantation for therapeutic angiogenesis in patients with ischemic vascular disease[39,40]. Surprisingly, the reduction in the number of EPC obtained from hyperglycemic BM was associated with a concomitant increase in the ability of these BM cells to generate Mph while the number of DC that could be obtained upon appropriate stimulation was not changed. We have previously shown that Mph and DC can be derived from common myeloid progenitor cell subsets in murine BM[25,26], and recent findings by Fogg *et al*. confirm this notion as they indicate that Mph and DC share a common BM progenitor[41]. Here, we observed that the frequency of the different myeloid progenitor subsets in BM was not changed by hyperglycemia. Therefore, we propose that myeloid lineage differentiation potential of the BM progenitors was skewed as a consequence of the hyperglycemic state. Our observations suggest that inflammatory cytokines or elevated redox signaling may directly regulate the differentiation of various myeloid lineages from progenitor cells. NF-κB is a major transcription factor responsive to cytokines and reactive oxidant species. Indeed, recent studies indicate that NF- κB, acting via IRF4, directly influences DC vs. macrophage differentiation[42].

Recently we demonstrated that EPC, Mph and DC show a significant phenotypic overlap and that a major source of EPC from the BM is the $CD31^{high}/Ly6C^{high}$ fraction that predominantly contains myeloid progenitor cells that can also generate Mph and DC[24]. This myeloid origin of EPC is consistent with the observation that $CD34^{low}CD14^{+}$ cells in peripheral blood are a major source of human EPC[21]. Assuming that the EPC, the Mph and the DC all originate from a common myeloid progenitor, we speculate that the pro-inflammatory milieu in diabetes not only skews the differentiation of the myeloid progenitor cell towards the Mph but that it does so at the cost of the generation of EPC. Several recent studies support this concept of heterogeneity of myeloid cells in response to environmental stimuli. In mice, diet-induced obesity was shown to induce a phenotypic switch of the adipose tissue macrophages from an anti-inflammatory towards a pro-inflammatory state[43]. Another study showed that a subfraction of circulating $Ly6C^{high}$ monocytes was selectively and progressively expanded under hypercholesterolemic conditions. This subfraction, compared to $Ly6C^{low}$ monocytes, displayed a highly pro-inflammatory and atherogenic phenotype[44]. As pointed out before, short-term cultured BM-derived EPC are mainly derived from a myeloid cell fraction with high levels of Ly6C. As EPC display a higher pro-inflammatory phenotype under hyperglycemic conditions, their possible role in progression of atherogenesis in this metabolically altered environment needs to be further explored. Our data imply that hyperglycemia-dependent alterations on cell-fate specification are not restricted to embryonic development[45] but may also affect the differentiation of adult progenitor cells. The ultimate proof of this concept may involve the use of techniques that monitor clonal expansion of the myeloid progenitor cells and HSC-like cells and are the subject of current investigations.

HMG-CoA reductase inhibitors, statins, have been shown to increase the number of circulating EPC both in animal models[32,33] as well as in patients with stable coronary artery disease[46]. Also, statins generate potent anti-inflammatory actions[31] and can improve properties of dysfunctional EPC populations *in vitro*[4,33,46]. In addition, statins appear to modulate the *in vitro* differentiation of monocytes to Mph[35]. Therefore, we assessed whether the addition of atorvastatin, *in vitro*, could reverse diabetes-associated changes in the EPC and Mph cultures. Indeed, we observed that atorvastatin restored EPC differentiation to control levels, and also strongly decreased the elevated number of Mph generated in the BM cultures from hyperglycemic mice. Also the angiogenic capacity of the EPC derived from hyperglycemic BM was partially recovered when cells were cultured in the presence of atorvastatin.

In conclusion, our study shows that EPC dysfunction in diabetes stems from the BM due to hyperglycemia-induced alteration in differentiation of myeloid progenitor cells. The inflammatory nature of EPC in hyperglycemia may not only impair EPC function but potentially also contributes to premature atherosclerosis when these cells incorporate into the vessel walls. Our observation that statins can, at least in part, counteract these effects may not only provide a helpful tool to elucidate the molecular mechanism underlying EPC dysfunction but may also contribute to the beneficial effect of statin therapy on the circulating levels of EPC in patients.

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Chapter 7

The PPARγ agonist pioglitazone is a potent transcriptional trans-repressor in both monocytes and endothelial progenitor cells of patients with type 2 Diabetes

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Abstract.

Myeloid cells have been identified as key players in the development of atherosclerosis and organ damage. While cells from myeloid origin have the potential to repair the vessel wall by differentiation into endothelial cells and by stimulating angiogenesis and arteriogenesis, they may also cause injury when differentiation into inflammatory phenotypes such as macrophages or dendritic cells occurs. It appears that inflammatory and metabolic stimuli determine the fate of myeloid cells. As PPAR agonists have been shown to have potent anti-inflammatory effects, we investigated whether short-term treatment with the PPARγ agonist pioglitazone modulates the inflammatory signature of circulating myeloid cells as well as the capacity to differentiate into an endothelial phenotype (EPC) in patients with type-2 diabetes. Transcriptional profiles of myeloid cells, committed to the monocytic lineage, as well as EPC were analyzed from patients treated for four weeks with pioglitazone and compared with patients who received placebo. A marked overall transcriptional repression was observed in differentially expressed genes of both monocytes (83.3%) and EPC (91.9%). Validation experiments with real-time PCR could further demonstrate that EPC's, in comparison to monocytes, show characteristics of immature immune cells with high C1q expression and low expression of the complement propagation factor properdin. Pioglitazone treatment shifted this profile in monocytes towards that of immature immune cells. Despite the transrepression, no difference in the ability of myeloid progenitor cells to differentiate towards endothelial cells was observed. This study provides the first *in vivo* evidence of transcriptional transrepression in myeloid cells in diabetes and points to a new therapeutic mode of action of thiazolidinediones.

Introduction

Over the last years myeloid cells have been identified as key players in the pathobiology of atherosclerosis and target organ damage. Circulating myeloid cells may differentiate into monocytes that subsequently can develop into foam cells when recruited into the vessel wall. Moreover myeloid cells also are a source of dendritic cells in the vessel wall[1]. This results in antigen presentation and local adaptive immune responses. Apart from these pro-inflammatory effects of myeloid cells, we and others recently found that myeloid progenitors may also differentiate into an endothelial phenotype and restore endothelial integrity[2,3]. Moreover, myeloid cells have been involved in lymphangionesis and clearance of inflamed tissue[4]. Inflammatory stimuli and cytokines seem to influence the fate and plasticity of such myeloid cells[5]. For example, in the presence of cytokines such as GM-CSF and interleukin-4 the formation of dendritic will be promoted[6]. The presence of VEGF and shear stress may facilitate endothelial differentiation[7,8] while VEGF-C and its receptor VEGF-3 have been shown to be a stimuli for formation of new lymph endothelium from these cells[4]. We previously demonstrated that diabetes is associated with a reduced capacity to form endothelial progenitor cells (EPC) from peripheral-blood mononuclear cells (PB-MNC) while function of these EPC was hampered as well[9]. In a recent study, we demonstrated that in mice hyperglycemia affects myeloid differentiation in the bone marrow resulting in a reduced differentiation of myeloid progenitor cells into EPC and a concomitant increase in the differentiation of myeloid progenitor progenitor cells into macrophages (in submission). Prevention of cellular cytokine responses and the transcription of inflammatory genes may therefore be a target to modulate the balance of myeloid cells towards repair and less to inflammatory phenotypes.

In the setting of diabetes, the synthetic PPARγ agonists are of particular interest in this respect. These drugs were designed and developed to induce PPARγ-dependent transcription in adipocytes, thus influencing differentiation and metabolic function of adipocytes[10]. This results in improved free fatty acid metabolism and enhanced glucose sensitivity[11]. However, more recently PPARγ agonists have also been shown to negatively regulate inflammatory gene expression[12-14]. The PPAR ligands exert these anti-inflammatory effects by inhibiting various transcription factors including NFκB[15].

In the current study we assessed whether the PPARγ agonist pioglitazone modulates transcriptional activation of myeloid cells as well as the capacity to differentiate into endothelial phenotype. For this purpose mononuclear cells that were committed to the

monocytic lineage and to the endothelial lineage, as well as late outgrowth endothelial progenitor derived cells, were characterized for their transcriptional signature. Subsequently, the transcriptomes of these cells were investigated in patients with type II diabetes, comparing pioglitazone treatment to placebo.

Subjects and methods

Patient characteristics.

Ten male, non-smoking patients with type 2 diabetes were recruited into this study. All patients were treated with oral antihyperglycemic agents that were continued during the study. Subjects with poor glycemic control (HbA1c > 9%) were excluded. Other relevant exclusion criteria were presence of macro- or microvascular disease and use of vasoactive medication (eg, [beta]-blockers, calcium entry blockers, ACE inhibitors, angiotensin type-1 receptor blockers, statins, aspirin, or non-steroidal inflammatory drugs). The ethical review board of the University Medical Center Utrecht (UMCU) approved the protocol. All subjects gave written informed consent. Measurements were carried out in accordance with local institutional guidelines in a Good Clinical Practice-certified unit[16].

Study design

The study was designed as a prospective, randomized, crossover, placebo-controlled, double blind trial. Patients eligible to take part in the study were randomized to receive pioglitazone 30 mg once daily (Eli Lilly) or placebo for 4 weeks in addition to their oral antihyperglycemic agents. These 4 weeks were followed by a washout period of 6 weeks. Peripheral blood was drawn at the end of each 4-week treatment period (placebo and pioglitazone). Patients were instructed to fast for at least 10 hours before the tests. No study medication or other medication was used on the morning of the study days.

Laboratory assessment

Fasting peripheral blood was drawn, and plasma was frozen at -20°C until further analysis. Glucose, creatinine, total cholesterol, triglycerides, and high-density lipoprotein cholesterol (HDL-C) were measured by standard enzymatic laboratory methods (Vitros 250; Johnson & Johnson). Low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula. HbA1c and free fatty acids (FFA) were photometrically performed (Hitachi 911;

Roche). Insulin levels were determined with an immunologic method (Immulite 2000; Diagnostic Products Corp). Measurements of plasma adiponectin, interleukin-6 (IL-6), and high-sensitivity C-reactive protein (hs-CRP) were performed with a commercially available kit (ELISA; R&D Systems Inc).

EPC isolation and characterization

Peripheral blood was obtained in blood collection tubes containing EDTA (Venoject). EPC were cultured as described[9]. Briefly, PB-MNC were isolated from 100 ml whole blood by density gradient centrifugation (Histopaque 1077). $50x10^6$ PB-MNC were plated at a density of 1 X 10^6 cells per cm^2 on 6-well culture plates coated with 2% gelatin (Sigma) in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics and 10 U/ml heparin (Leo Pharma BV). After four days of culture, EPC characteristics were confirmed on the basis of morphology and by fluorescent confocal immunohistochemistry using Ulex europaeus agglutinin (UEA-1: Vector), a CD31 antibody (DAKO Diagnostics) and DiI-labeled acetylated LDL (Molecular Probes). EPC were isolated, counted and stored at –80°C as cell pellets until use.

To culture late-outgrowth endothelial cells with a cobblestone like morphology (CLC), MNC fractions of cord blood were isolated and cultured as short-term EPC (see above). After 7 days medium was replaced with EBM endothelial cell medium supplemented with bulletkits (Cambrex). Cells were cultured for over 4 weeks, until colonies with cobblestone morphology appeared. When they reached confluence, the cells were passaged for maximal 4 times in gelatin coated culture flasks. Before use, the CLC were analyzed by immunofluorecence techniques staining for mature EC characteristics with vWF (DakoCytomation), Endoglin (BD, transduction Laboratories) and eNOS (Pharmingen) antibodies.

Human Umbilical cord Vein Endothelial Cells (HUVEC) were also cultured in EGM medium on gelatin-coated culture flasks up to four passages. Total RNA was isolated using an RNeasy kit (Qiagen) and the integrity of RNA preparations was validated before use.

Monocyte ($CD14^+$) isolation by magnetic cell sorting

$CD14^+$ cells were purified from PB-MNC (see above) by positive selection with magnetic beads conjugated to anti-CD14 antibodies (Miltenyi) using an autoMACS system and according to the manufacturers protocol. $CD14^+$ monocyte fractions were reproducibly over

95% pure as determined by flowcytometric analyses (FACScan) and were stored at -80°C for protein (whole cell lysate) and/or total RNA isolations (Rneasy kit, Qiagen).

In vitro angiogenesis assay.

To evaluate CLC cells for their capacity to form tube-like structures, $0.7*10^5$ cells/cm^2 passage 4 CLC were seeded on human fibrin matrices and cultured in M199 medium supplemented with 10% human serum, 10% newborn calf serum, penicillin/streptomycin, basic fibroblast growth factor (bFGF) and tumor necrosis factor- (TNF-)17. Fresh medium was added every second day and the appearance of invading cells and tubular structures was evaluated by phase-contrast microscopy 7 days after seeding.

Microarray analyses

For gene expression profiling of $CD14^+$ cells, EPC cultured for 4 days, late-outgrowth EC (CLC) and HUVEC, total RNA preparations were obtained and analyzed using Affymetrix high-density HG-U95Av2 oligonucleotide microarrays interrogating 12.600 transcripts, according to the manufactures protocol. To reduce individual-specific variation in gene expression, $CD14^+$ and EPC RNA samples were derived from a pool of equal amounts of total RNA from 5 (healthy) volunteers. Also CLC and HUVEC RNA was derived from 5 donors and pooled.

For gene expression profiling of $CD14^+$ and four-day EPC cultures obtained from patients with type-2 diabetes, messenger RNA profiles were analyzed using Affymetrix high-density HG-U133A oligonucleotide microarrays interrogating 18.400 transcripts. Equal amounts of total RNA from 5 patients with either placebo or pioglitazone treatment were pooled.

All the scanned images were analyzed using Affymetrix Microarray Software (MAS) and significant differentially expressed genes (placebo as background signal) were further analyzed. Scaling factor, background, noise and 3'/5' GAPDH ratios (always less than 1.2) were such that valid comparisons between similar arrays could be made. For further analyses commercially available programs Matlab and Spotfire were used.

Quantitative rt-PCR

For validation of the microarray analyses, mRNA expression levels of C1q, Factor P, CDKN1A and S100A9 and two normalization genes (Actin and GAPDH) were measured using quantitative RT-PCR. cDNA was synthesized from total RNA samples (same as used for profiling) using standard cDNA synthesis reagents and a 1:1 mixture of oligo dT(12-18)

primers and random hexamer primers (Invitrogen). Quantitative analyses of the synthesized cDNA were performed with use of SYBR green I (Molecular Probes) in optimised real-time PCR (Amplitaq Gold, Applied Biosystems), using a iCycler Thermal cycler (Biorad). Gene specific primer combinations were assembled with Oligo Explorer (Gene link). Primer sets of the various genes used were from 5' to 3': C1q forward: tcaccaaccaggaagaaccg, reverse: atgggaagatgaggaagccg. Factor P (Properdin) forward: cctaatcctacccgtgcc, reverse: cttctcgccctgaccttc. Cyclin-dependent kinase inhibitor-1A (CDKN1A) forward: gattagcagcggaacaagg, reverse: caacgttagtgccaggaaag. S100A9 forward: gctggaacgcaacatagag, reverse:ggtcctccatgatgtgttc. For normalization, GAPDH forward: ttccaggagcgagatccct, reverse: cacccatgacgaacatggg and actin forward: tgcgtgacattaaggagaag and reverse:tgaaggtagtttcgtggatg. Samples were analyzed in triplicate and threshold cycle numbers and their SD were calculated using iCycler v3.0a analysis software (Biorad) and further used to calculate expression ratio's of the different samples in relation to both normalization genes.

Results

Patient characteristics

Ten subjects were included in this study. No carryover effects between the 2 treatment periods were observed for any parameter[16]. When treatments of either placebo or pioglitazone were compared (table 1) a significant reduction in FFA was observed (640±38 versus 504±34 μmol/L, P=0.04; respectively. A significant increase in adiponectin plasma levels was obtained during pioglitazone treatment compared with placebo after only 4 weeks (7421± 147 versus 4264±856 ng/mL, P=0.005 respectively). No further significant differences were seen in the measured values.

$CD14^+$ monocyte isolation and EPC characterization

To assess the myeloid properties and the purity of the sorted $CD14^+$ cells the cells were stained with an anti-CD14 FITC-labeled antibody and analyzed by flowcytometric analysis. Figure 1A shows a representative dot plot of the sorted $CD14^+$ fraction that was reproducibly over 95% pure (96.5% ± 2.4%, n=10). After 4 days of cultivation under EC differentiation conditions, attached cells were analyzed for EC characteristics. EPC appeared with a typical spindle-shaped morphology (figure 1B, upper right) and over 90%

	Placebo (n=10)	Pioglitazone (n=10)	P-value
Weight (kg)	99.3 ± 3.0	98.6 ± 3.9	0.71
SBP (mm Hg)	137 ± 7.0	130 ± 3.0	0.44
DBP (mm Hg)	73 ± 2.0	74 ± 3.0	0.90
Fasting glucose (mmol/L)	7.2 ± 0.5	6.6 ± 0.6	0.25
Insulin (mU/L)	21.0 ± 4.2	17.1 ± 3.3	0.33
HbA1C %	6.7 ± 0.2	6.4 ± 0.2	0.08
Total cholesterol (mmol/L)	4.8 ± 0.2	4.9 ± 0.3	0.34
HDL cholesterol (mmol/L)	0.9 ± 0.05	0.9 ± 0.1	0.92
LDL cholesterol (mmol/L)	3.0 ± 0.2	3.1 ± 0.3	0.57
Triglycerides (mmol/L)	1.8 ± 0.2	1.8 ± 0.2	0.72
Free fatty acids (μmol/L)	640 ± 38	504 ± 34.0	0.04 *
Adiponectin (ng/ml)	4264 ± 856	7421 ± 1147	0.005 *
Creatine (μmol/L)	71 ± 3.0	69 ± 2.0	0.11
CRP (mg/L)	3.9 ± 0.8	2.7 ± 0.6	0.11
Interleukine-6 (pg/ml)	2.0 ± 0.3	1.7 ± 0.2	0.17

Values are mean ± SEM.
P-values calculated Placebo vs. Pioglitazone.
*P<0.05

Table 1
Clinical characteristics of the patients after treatment

of the attaching cells showed uptake of DiI-labeled acLDL (red) and could bind an EC specific lectin UEA-1 (figure 1B, upper left). Further characterizations show that EPC comprise more EC properties as they have a high CD31 surface expression and they express low levels of eNOS. Von Willebrand factor was also detected at low levels in the EPC although no Weibel Pallade body-like structures could be observed (data not shown). Typical clonal cell expansion is seen in the late-outgrowth cultures (CLC) after 4 weeks of culture (figure 1B, middle left). These colonies were further cultured until they formed a monolayer of cells with cobblestone morphology. Endothelial characteristics of these cells were analysed and confirmed using immunofluorescent staining of vWF (in Weibel Palade bodies, figure 1B, middle right), CD31 (figure 1B, lower right), eNOS (mostly perinuclear staining) and Endoglin (data not shown). To assess whether the CLC also display functional

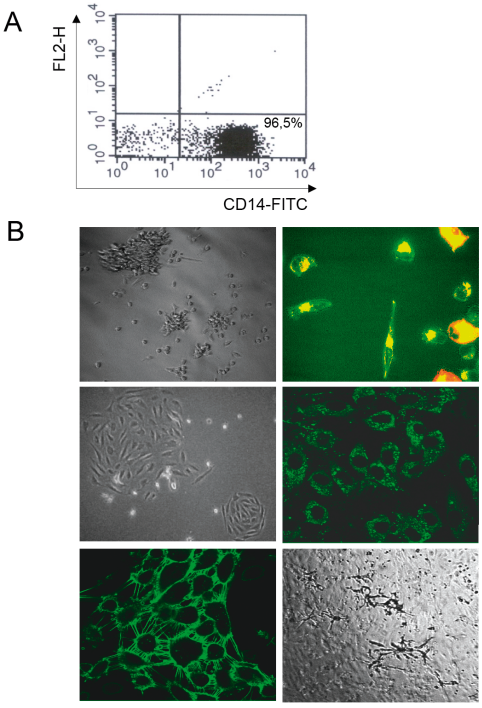


Figure 1: Morphological, phenotypical and functional analyses of the different cell populations.

(A) Flowcytometric analyses of the purity of $CD14^+$ cells in the positive cell fraction after magnetic cell sorting.

(B) Morphology of cell clusters of EPC appearing after 4 days (upper left). Upper right shows uptake of Di-labeled acLDL(in red) by EPC cultured for 4 days as well as binding of lectin UEA-1 (in green). Almost all cultured cells are double positive for both EC markers. When EPC are cultured further as CLC they start out as colonies with cobble-stone appearance (middle left panel). CLC grown as monolayer all show vWF staining in Weibel pallade bodies (middle right panel) and CD31 membrane staining (lower right) similar to mature EC. Furthermore, CLC are able to form 3-D vessel structures in *in vitro* angiogenesis assays (lower right panel).

EC characteristics, the ability of these cells to form vessel like structures in an *in vitro* angiogenesis assay was examined. Indeed, CLC readily formed capillary-like structures to the same extend as mature EC upon stimulation with angiogenic factors like bFGF (figure 1B, lower right).

To demonstrate the myeloid lineage properties of the isolated $CD14^+$ cells and the myeloid / EC character of the cultured EPC, mRNA profiles were analyzed for monocyte and endothelial cell specific gene expression (table 2). Genes that are associated with monocytes are shown at the top of the table, while genes known to be EC specific are depicted at the bottom. The middle represents a transition phase, as monocytes and EC are known to share some antigens. The table validates the myeloid character the sorted $CD14^+$ cells reveals that EPC share many genes with these myeloid $CD14^+$ cells and have only upregulated some EC specific genes (e.g. endoglin) at a low level. These data further support the concept that short term cultured EPC are derived from the myeloid lineage. Consistent with the phenotypic and functional analyses of the CLC, profiles of the late-outgrowth EPC (CLC) show a high similarity in gene expression with mature EC (HUVEC).

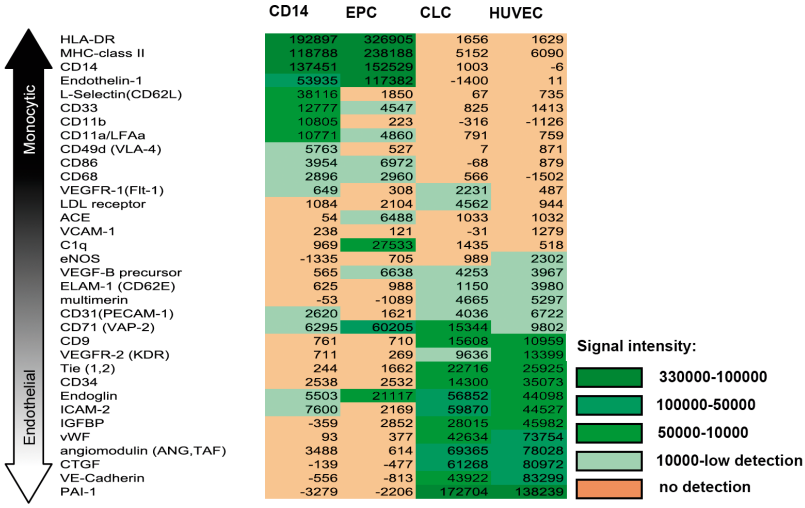


Table 2

Myeloid/Endothelial characteristics of CD14$^+$ cells, EPC, and CLC. Micro array analyses were screened for monocytic and endothelial genes and expression (laser signals) of these genes are depicted as high (dark green), intermediate (light green) and low or below detection (orange).

Pioglitazone treatment does not induce differences in the number of monocytes or EPC

To investigate whether pioglitazone treatment did affect the number of circulating CD14$^+$ cells, the purified CD14$^+$ fractions derived from equal amounts of MNC fractions were quantified. As shown in figure 2A, no significant difference in CD14$^+$ cells numbers were observed. Pioglitazone treatment did also not have an effect on the differentiation of myeloid cells into the EC lineage, as the number of attaching EPC cells (4 day cultures) derived from 50x10^6 MNC was similar compared to the cultures from placebo treated patients (figure 2B).

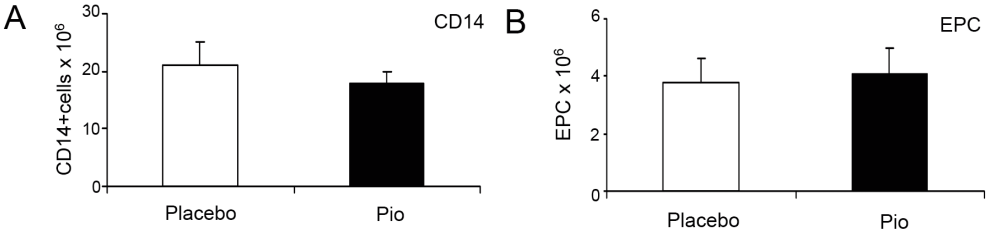
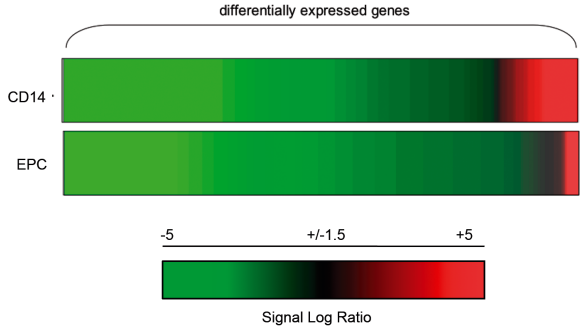


Figure 2: Effect of pioglitazone treatment on number of cells.

The treatment of the patients with pioglitazone (Pio) had no significant effects on the total number of isolated CD14$^+$ cells (A) and the total number of cultured EPC at day 4 (B) when compared to placebo treatments.

Transrepression of differentially expressed genes by pioglitazone treatment.

As the overall average expression of the raw data signals of all microarrays were comparable (data not shown), the effects of different treatments on the two different cell types could be compared using MAS microarray analyses. Significantly differentially expressed genes (1.5x cut-off value) in $CD14^+$ cells and in EPC were extracted from the panel and their Signal Log Ratio's were plotted (figure 3). There were more significant differentially expressed genes in the EPC panel when compared to the $CD14^+$ cell panel (1178 genes vs. 514 respectively). Strikingly, almost all differentially expressed genes were down regulated in both $CD14^+$ cells and EPC (83.3% and 91.9% respectively), suggesting a highly dominant transrepressive effect of pioglitazone treatment in subsets of myeloid cells derived from diabetes patients.



	CD14	EPC
# genes	514	1178
% increased	16,7	8,06
% decreased	83,3	91,9

Figure 3: Transrepression of differentially expressed genes occurred in both $CD14^+$ cells and EPC in patients treated with Pioglitazone.

Genetic profiles of both pioglitazone and placebo treatments (background values) are compared of $CD14^+$ cells and EPC and only the differentially expressed genes are depicted.

Signature of inflammatory responsive genes by pioglitazone treatment.

In order to evaluate the effect of pioglitozone treatment on inflammatory responsive genes in both cell types we looked for the genes present on the HG-U133A that represent the gene ontology class "Immune response" (GO:00069). We extracted about 50 different probe sets from this GO-class and we calculated the ratios of the probe signals (placebo= background). By plotting these ratios, the fingerprint of this specific GO-class of genes became visible (figure 4).

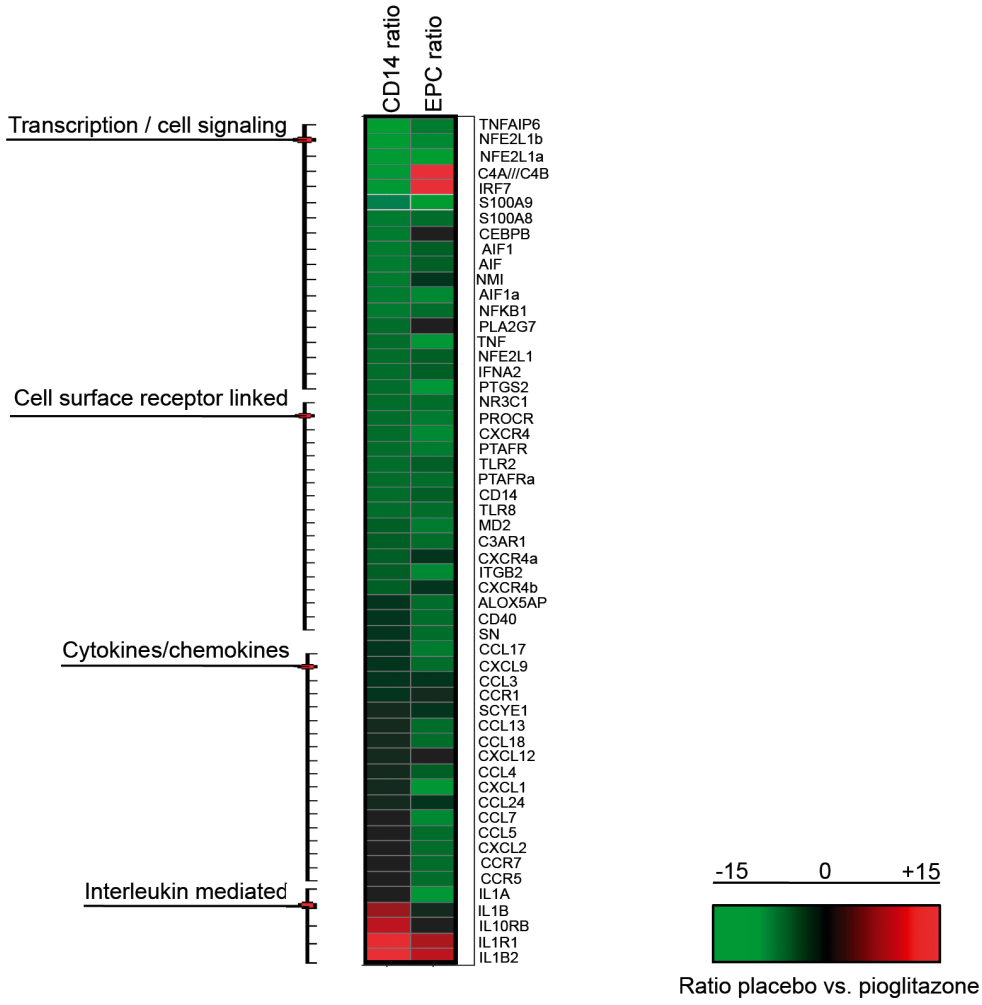


Figure 4: The effect of pioglitazone treatment on inflammatory responsive genes in CD14⁺ cells and EPC.

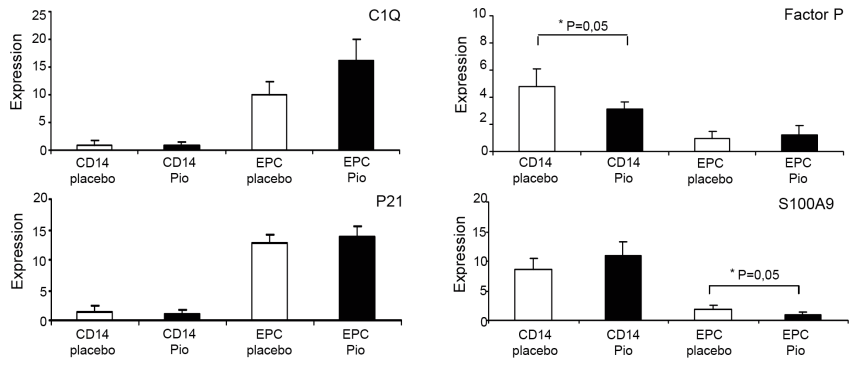
Some of the genes are represented several times in the figure but different probe sets were analysed for these.

Validation of microarrays by quantitative real time PCR analyses

To validate if the gene arrays were representing the mRNA levels in the different cells, four genes of interest were analyzed by quantitative rt-PCR. Expression ratio's of the different genes were normalized to two different normalization genes (GAPDH/Actin) and results are shown in figure 5A. Raw data signals from the various genes (figure 5B) were assembled from the array analysis and compared to the expression data derived from the

quantitative rt-PCR. Complement factor C1q was analyzed in the patient samples (figure 5A) as well as in the various differentiation stages of endothelial cells (figure 5C). C1q, a major component of the classical complement pathway, was not significantly differentially regulated by treatment of pioglitazone in both cell types (figure 5A); however it showed a marked upregulation in the EPC differentiation stage when compared to monocytes or mature EC (figure 5C). Quantitative analyses of factor P mRNA levels, also known as Properdin, a positive regulator of complement activation showed marked higher levels in $CD14^+$ cells when compared to EPC. No significant differential expression after pioglitazone treatment was observed in EPC, however in $CD14^+$ cells we found a marked reduction in Factor P (P = 0.05). P21 mRNA levels showed the same pattern as mRNA levels of C1q in the patient samples, revealing no significant differential expression in both

A



B

	CD14 Placebo	CD14 Pio	EPC Placebo	EPC Pio
C1Q	2019	6069	36186	29210
Factor P	38629	18874	6201	5360
P21	4222	5049	25224	17737
S100A9	402387	336445	79624	49101

C

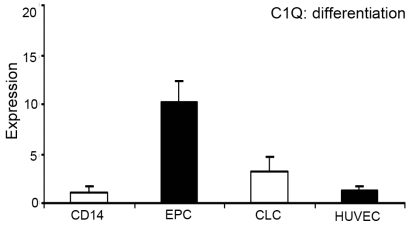


Figure 5: Validation of microarrays by quantitative real time PCR analyses.
Gene expression profiles of CD14 cells and EPC derived from patients with (Pio) or without (placebo) pioglitazone treatment were validated. Normalized relative expression ratio's of mRNA levels of C1q, Factor P, P21 and S100A9 were analyzed using quantitative real time PCR techniques (A). Raw data signals of the microarrays were plotted to be able to compare these expression levels with the expression ratio's generated by quantitative PCR (B). In addition to the patient samples C1q profiles of various differentiation stages of endothelial cells were further explored using quantitative PCR. Table 2 shows the raw data signals of the microarray experiments and here these data are verified (C).

cell types when patients were treated with pioglitazone but a marked increased expression in EPC when compared to CD14 cells. When we quantified S100A9, a Ca^{2+}-binding protein known to be involved in inflammatory responses of monocytes and macrophages[18], we noticed that $CD14^{+}$ cells had a higher expression of the protein when compared to EPC but EPC showed a significant reduction in the level of S100A9 after pioglitazone treatment ($P = 0.05$).

In summary, for all genes tested, observations obtained by quantitative rt-PCR were in line with the microarray analyses, showing that the gene expression levels from the microarray analyses did represent mRNA levels in the cell isolations.

Discussion

Recently attention has been drawn to the anti-inflammatory and anti-atherosclerotic effects of synthetic PPAR-y ligands. The current study provides a rationale for these effects by demonstrating generalised trans-repression in myeloid cells that are involved in inflammation and repair processes in patients with type II diabetes by the PPAR-y agonist pioglitazone.

The classical paradigm is that thiazolidinediones, synthetic ligands for PPARγ, act through transcriptional activation in adipocytes resulting in improved free fatty acids metabolism and glucose handling[10,11]. However, in recent years data have emerged that also show direct vascular effects of these drugs. For example inhibition of atherosclerosis progression was observed in non-diabetic patients[19]. It was suggested that this effect was independent of changes in metabolism and could possibly be attributed to anti-inflammatory effects. Indeed, thiazolidinediones have been shown to reduce tissue inflammation in conditions such as psoriasis and kidney disease[20]. The exact nature of these effects is not exactly known yet.

The current study shows that patients with type 2 diabetes that have been treated with pioglitazone have a generalised transcriptional repression in circulating monocytes (figure 3). This trans-repression includes important genes involved in cell activation such as cell surface receptor linked mechanisms, the cytokine and chemokine production and genes involved in control of transcription (figure 4). It has been proposed that such trans-repression may be secondary to a physical interaction between PPAR-γ and the co-repressor complexes that keep inflammatory transcription suppressed[15]. Binding of PPAR-γ to this

repressive complex prevents recruitment of the ubiquitination machinery normally required to clear the co-repressor complex from the activation site. The current study extrapolates these *in vitro* concepts to the *in vivo* situation in patients with type 2 diabetes. As monocyte activation and transformation into foam cells is fundamental to atherogenesis and plaque disruption, one may postulate that these molecular effects of thiazolidinediones contribute to the beneficial effects on atherosclerotic disease of these drugs. Indeed, reduced progression of intima media thickness, as an intermediate marker of atherosclerosis, has been observed both in diabetic patients[21] as well as in non-diabetic patients[19]. Moreover in a recent large endpoint driven study, the PRO-active study, it was shown that pioglitazone could produce cardiovascular events and associated mortality[22].

The present short-term study only shows very limited effects on metabolic indices. Only small changes in free fatty acid fluxes could be observed, while glucose and lipid levels did not change significantly. For metabolic changes the treatment period could be too short as they are generally only seen from 8 weeks of treatment[20]. This makes a direct transrepressor effect by pioglitazone more likely than an indirect effect through improvement of insulin resistance.

We recently could demonstrate that myeloid cells may not only give rise to inflammatory cells but are also implicated in repair processes of the vessel wall. For example they have been shown to be able to reendothelialize[2] and to enhance angiogenesis[23,24] and lymphangiogenesis[4,25]. The current study shows that despite the generalized trans-repressive effects of pioglitazone, differentiation into an endothelial cell phenotype is still possible. The number of endothelial like cells (general referred to as endothelial progenitor cells, EPC) that can be cultured from the mononuclear cells is the same whether the patients are treated with placebo or with pioglitazone (figure 2B). Nevertheless when these cells are in the process of transdifferentiation towards an endothelial phenotype they show a similar profound reduction in expression of genes that are involved in cell activation. Previously, using a mice model it was observed that thiazolidinediones might even increase the capacity to form endothelial cells from bone marrow. It should, however, be noticed that these were healthy mice that did not have diabetes-associated recruitment ad differentiation defects[26].

We also characterised the expression profiles of myeloid cells while they are in transition of myeloid to endothelial cells. When cells are cultured for 4 days under conditions that promote the formation of endothelial cells, expression of endothelial specific genes such as

endoglin, VEGF-B become upregulated (table 2). However these cells still bear more characteristics of myeloid cells. For example they express HLA-DR and CD14. Only after prolonged culture a full endothelial phenotype (CLC) with a similar expression signature to that of e.g. HUVEC appears. This would imply that the early re-endothelializing cells still could be considered as cells that are part of the innate immune system. In this respect they demonstrated some interesting features that do distinguish them from monocytes. For example the complement factor C1q is strongly upregulated. (figure 5C, table 2). Recent data from our laboratory have shown that C1q produced by myeloid cells acts as an immune-modulatory factor that allows for phagocytosis and clearance of apoptotic cells but blocks T-cell activation[27,28]. This suggests that these EPC also participate in immune surveillance. It is of interest to notice that treatment with pioglitazone upregulates C1q in the monocytes while the monocyte complement propagation factor porperdine, which allows for full complement activation, is downregulated. In this respect pioglitazone treatment shifts the expression in monocytes of diabetes patients towards that of immature immune cells.

In sum, our study provides first *in vivo* evidence of a novel mode of action of thiazolidinediones that may contribute to organ protection in diabetes.

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Chapter 8

Conclusions, General Discussion and Future Directions.

Cindy J.M. Loomans

8.1 Conclusions, general discussion

As described in chapter 1, EPC are bone-marrow derived cells that participate in postnatal neovascularization. Once they are mobilized into the periphery they home towards injured or hypoxic areas to facilitate angiogenesis and vasculogenesis. Postnatally, EPC are needed to maintain the integrity of the endothelium (reendothelialization) and to augment wound healing or to vascularize hypoxic areas (neovascularization). In patients with classical risk factors for ischemic vascular disease, EPC numbers have been shown to be reduced and their function has been shown to be impaired. The work presented in this thesis explored EPC in healthy subjects and focused on their altered properties under hyperglycemic conditions using cells from patients with diabetes mellitus and cells of STZ-induced diabetes in mice.

The nature of EPC

Although various EPC containing cell preparations have already been explored for therapeutic application, to date, EPC are poorly characterized and the nature of the optimal cells for therapy is unknown. As many groups use different methods and surface markers to isolate these cells the studies are hard to compare. Especially for short-term cultured EPC the different protocols use different growth factors and adhesive surfaces. To my opinion, to date there are no distinct markers to identify true EPC, that rule out mature EC and or other myeloid lineage cells like DC and Mph in both human or wild-type mouse. This makes it hard to distinguish and study endothelial progenitors and their sources. For that reason, the data described in chapter 5 are important as a they introduce a transgenic mouse model, in which GFP is placed before an EC-specific human eNOS promoter[1]. Although, some groups have described eNOS protein in alveolar lung-derived Mph[2,3], we could not show any eNOS expression in mature monocytes, Mph or DC derived from bone marrow cells cultured under various conditions. Therefore, this transgenic mouse model gives us and other researchers a valuable tool to further explore origin and function of BM-derived EPC cultures under different experimental conditions. In chapter 5, mostly BM-derived EPC cultures were studied but when we used the spleen as another source of EPC, we saw that progenitor cells from the different origins reacted different to various growth stimuli suggesting a “different” nature of the EPC-like progenitors in the periphery when compared to those cultured from the progenitors in the BM. This intriguing finding is important and

needs to be taken in consideration when determining which progenitor fraction is best to use for cell therapies.

Another aspect of EPC that has not been described and understood yet is the differentiation lineage of EPC. The study in chapter 5 shows us that short-term cultured EPC are mainly derived from a specific myeloid precursor fraction of the BM, with an abundant expression of both CD31 and Ly6C on their surface. This is in line with several studies that show monocytic features on the progenitors of EPC[4-7]. Taken together our data for short-term mouse bone marrow derived EPC, supports a myeloid transition phase in the differentiation of EPC toward the mature endothelial cell. However, if EPC also can differentiate from "full" monocytes is not known. In chapter 5 we describe that we failed to culture EPC from a more mature monocyte population ($Ly6C^+$, $CD31^-$) of murine BM suggesting that there is a less mature myeloid progenitor cell that still has the potential to develop into the EC-lineage. This is somehow unexpected as other groups have shown that circulating human EPC can be derived from "mature" circulating $CD14^+$ monocytes.

It has to be taken into consideration that Mph and DC can be cultured from the same myeloid progenitor fraction and similar to EPC, osteoclasts can be solely cultured from this same fraction (data Leenen *et al*, not published). It is not completely sure if all these myeloid derived cells are truly derived from the same fraction or if the $CD31^+/Ly6C^+$ fraction contains different subfractions of progenitors. Mph and DC likely do have the same progenitor as switching different growth factors *in vitro* can completely shift the fate of the total population to the desired population of cells[8]. Recently, other studies described a common progenitor for adipocytes and endothelial cells[9], showing an even wider diversity and plasticity of the differentiation lineage of EPC. These data indicate a tight regulation of the differentiation of myeloid progenitor cells, a topic that needs to be further explored. In particular, the use of clonal assays may give answers to the relation of these the myeloid lineage derived phenotypes.

Using the eNOS-GFP transgenic mouse model, we were able to look at the heterogeneity between different mouse strains and a marked difference was observed in the capacity of BM-progenitors from C57BL/6J and FVB mice to differentiate into EPC. In particular in FVB mice a 40% increase in the total number of EPC derived after 7 days culture was shown. Given the fact that FVB mice are much less susceptible for atherosclerosis compared to C57BL/6J mice, one could speculate about the importance of the capacity to form EPC in a relative short time. This heterogeneity may also be observed in humans, however because of a lack of good distinct EPC markers it is hard to reveal this difference.

Function of EPC

EPC have been shown to enhance neovascularization in many preclinical and *in vitro* studies[10-12]. In this thesis, a couple of additional insights in the function of EPC are presented. Chapter 2 describes a model that can dissect different mechanisms involved in neovascularization like migration, proliferation, differentiation, stimulation of, and integration in capillary-like structures. With an *in vitro*, 3-dimensional angiogenesis model we were able to study the effect of different subsets of EPC on neovasculogenesis. Early HSC $CD34^+$ cells were used and compared to cultured EPC, previously shown to be mainly derived from myeloid $CD34^-$ fractions[7]. $CD34^+$ cells were shown to home to sites of neovasculogenesis and proliferate at site. Furthermore, a small part of the $CD34^+$ cells did differentiate and incorporate in the mature EC monolayer and these cells were shown to enhance neovasculogenesis. However, $CD34^-$ cells could enhance this neovasculogenesis even further and to a similar extend as cultured EPC indicating that interactions between $CD34^+$ cells and $CD34^-$ cells can contribute to stimulation of capillary growth. Many studies have suggested an angiogenic paracrine effect of $CD34^-$ cells[5,6].

Another study describes that incorporation of $CD34^-CD14^+$ cells in to capillary-like structures requires co-injection with $CD34^+$ cells, indicating that direct leukocyte-leukocyte interactions may play a critical role in *in vivo* neovascularization[4]. Studies and models as presented in chapter 2 can give more insight in cell-cell interactions, diverse mechanisms of neovasculogenesis and different functions and capacities of the various subsets of EPC in future experiments.

Data presented in chapter 7 suggest that EPC may also function as cells involved in immune surveillance as gene expression data reveal that early cultured EPC (that express many myeloid cells markers) highly express immunoregulatory genes like C1q when compared to monocytes or mature EC. C1q is a component of the complement system which is designed to allow rapid and efficient activation and clearance of either foreign targets or altered/apoptotic/necrotic cells[13]. Furthermore, C1q has been implicated in preventing autoimmunity and maintaining tolerance by modulating professional phagocytes by for instance regulating IL-12p40 production and reducing NFκB activity[14]. The latter has been studied in immature dendritic cells derived from BM. If the same mechanisms play a part in BM-derived EPC still needs to be further explored. The finding that EPC do produce IL-12p40 (chapter 5) further supports the idea that these cells could regulate immune responses. These responses could imply another important role for EPC in process like wound healing.

EPC in hyperglycemia

At the onset of the studies described in this thesis no such thing as EPC dysfunction had been reported. Our hypothesis that EPC might be dysfunctional under hyperglycemic conditions arose from the finding that diabetic conditions could severely impair endothelial function and induce ischemic vascular events[15]. If EPC are really so important in vascular maintenance and repair there could be either less or dysfunctional EPC in Diabetes Mellitus, contributing to the pathogenesis of vascular disease in these patients.

Effect of hyperglycemia on the number of EPC

In chapter 3 it is described that we indeed observed a 40% reduction in the circulating number of short-term cultured EPC in patients with type 1 diabetes. This finding was directly related to the severity of the disease as we demonstrated a significant inverse correlation between the number of EPC and glycemic control (HbA1C). Similar results were also found in a hyperglycemic mouse model (chapter 6), further supporting the hypothesis that indeed hyperglycemia could affect EPC numbers. Likewise, Tepper *et al* found a reduced number of EPC in type 2 DM patients, however these patients, next to hyperglycemia, could also have other prevalent risk factors for EPC dysfunction[16]. Many pathophysiological mechanisms could account for this reduction in the number of EPC including impaired mobilization, impaired differentiation, increased apoptosis or cell senescence, reduced adherence capacities or exhausted pools of progenitor cells. Impaired mobilization due to a lack of eNOS has for instance been described[17]. Although, no reduction in the total number of circulating $CD34^+$ cells in diabetes patients was observed[18,19], a closer look at the distribution of different subsets of $CD34^+$ cells (KDR^+) might provide more insight in this matter. Altered differentiation could well be a mechanism involved in lower numbers of EPC as chapter 6 describes a possible skew of BM myeloid progenitors towards the macrophage lineage. Next to the reduced number of EPC, we observed an increased number of Mph that also related to the glycemic control of the mice. We speculate that the altered milieu in diabetes not only directs differentiation of progenitor cells towards Mph but that it does so at the cost of the generation of EPC.

Effect of hyperglycemia on the function of EPC

Chapter 3 not only describes a reduced number of EPC in type-1 DM patients but also that EPC isolated from the diabetes patients have an impaired angiogenic capacity compared to healthy control subjects. A similar observation in STZ-induced diabetic mice is described in

chapter 6 also revealing an impaired paracrine angiogenic function of hyperglycemic EPC. In addition, these dysfunctional EPC also display higher pro-inflammatory capacities (chapter 6). By using gene expression profiles (chapter 4), we show that human EPC from type 1 DM patients upregulate numerous pro-inflammatory genes known to be associated with hyperglycemia and oxidative stress. The pro-inflammatory nature of EPC in type 1 DM patients is a concern as they may contribute to an adverse (immune) response that may be pro-atherogenic and contribute to progressive ischemic vascular disease. This may be particularly relevant when using autologous EPC for therapeutic purposes. EPC are thought to function in sites of ischemia or reperfused tissue, which can be characterized as an inflammatory site. Inflammatory sites are known to be a high oxidative stress environment, which could be another mechanism for EPC dysfunction as described in chapter 4[20]. Given the central role of oxidative stress in type 1 diabetes, oxidative stress or altered redox signaling may also directly affect the survival, differentiation and function of EPC. As described in chapter 4, another potential mechanism for altered EPC function is the hyperglycemia associated formation of intra- and extra-cellular advanced glycation endproducts (AGE). Differential gene expression profiles support these hypotheses as we observed a striking number of genes upregulated in the EPC from type 1 DM patients that have been have been reported to be associated with diabetes mellitus in general, with hyperglycemia, oxidative stress or AGE formation. It is somehow remarkable that these different gene profiles, and consequently altered functional behavior, of EPC derived from either diabetic patients or hyperglycemic mice are found as these EPC are cultured for either 4 (chapter 3, 4 and 7) or 7 days (chapter 6) under normoglycemic conditions. So somehow EPC are “biosensors” that translate metabolic cues into altered gene expression partially imprinted for at least a week.

EPC in therapeutic applications.

Since the discovery of EPC, the cells have been of great interest as therapeutic entities. As mobilization of progenitor cells after injury, an important natural response to vascular injuries[21-23], did correlate with the long term outcome of cardiovascular disease[24] autologous cell transplantations became relevant. Over the last years, many promising preclinical studies have shown evidence that transplantation of different angiogenic cell populations did indeed contribute to repair of vascular injuries[25,26]. A series of clinical pilot studies, using cell transplantation in vascular diseases yield promising data[27-29], however some larger trails and longer follow-up studies did not reveal any benefits[30] or some very

minor benefits as discussed in chapter 1. It needs to be pointed out that treatments of myocardial infarction were less promising than treatments of in critical ischemia of extremities[28,31]. So far, most studies have mainly focused on unselected mononuclear cell fractions of bone marrow or peripheral blood. A recent clinical study showed that intracoronary administration of early hematopoietic $CD133^+$ BM derived cells in patients with recent myocardial infarction was associated with increased incidence of coronary events[32]. So although cell transplantation was associated with improved heart function there were unexpected side effects when using these early progenitor cells.

These and other discouraging outcomes sharpen the field of progenitor cell use for vascular therapy a bit and make researchers think about major questions like: what subset of cells should we transplant, an early progenitor or a further differentiated and maybe more dedicated cell? And what are the exact mechanisms these cells work by? To my opinion these questions have to be addressed first before further clinical trails can be initiated.

The work presented in this thesis gives insight into some of these questions. Chapter 5 shows a BM specific fraction ($CD31^+/Ly6C^+$) from which EPC could be derived but it also reveals a very high plasticity in this precursor fraction. This high plasticity of early progenitor cells could be a problem in cell transplantation therapies in general as precursors could differentiate to unwanted phenotypes depending on local differentiation/growth factors and inflict unwanted side-effects.

Chapters 3 and 6 show EPC dysfunction in type 1 diabetes patients and in hyperglycemic mice respectively. These EPC dispose dysfunctional properties in their angiogenic capacities (chapter 3 and 6) but they also reveal a higher inflammatory phenotype (chapter 6). Furthermore, our data implicate a possible skew of myeloid progenitors towards macrophage-like phenotypes. It can not be excluded that transplantation of these autologous myeloid progenitor cells would inflict adverse side effects. Chapter 6 and 7 suggest possible mechanisms to (partially) overcome these effects with statin interventions (Chaper 6, *in vitro*) or with PPARγ agonist interventions (chapter 7, *in vivo*). HMG-CoA reductase inhibitors have been shown to increase the number of circulating EPC[19,33,34] and they have been shown to generate potent anti-inflammatory actions[35] and can improve properties of dysfunctional EPC populations *in vitro*[19,34,36]. In addition, statins were shown to reduce the *in vitro* differentiation of monocytes to Mph[37]. Indeed we find that *in vitro* addition of atorvastatin (chapter 6) increases the number of EPC and conversely decreases the number of macrophages (*in vitro*). Statins could also recover the altered angiogenic properties of the EPC cell population derived from hyperglycemic mice.

Short treatment of diabetic patients with PPARγ agonists do show transrepression of many genes involved in inflammation, which might be another option to decrease the inflammatory phenotype of EPC.
Optimal treatment of diabetic patients to overcome EPC dysfunction and its side effects would still first be controlling the HbA1C levels of the patients and secondly find adjunctive therapy like statin/PPARγ agonist interventions to improve EPC functions.
Recently an interesting paper appeared discussing the potential to augment experimentally-induced ischemia of peripheral $CD34^+$ - and monocytic-like $CD14^+$ endothelial cell progenitors in diabetes[38]. The same group had found before that $CD34^+$ cells were dysfunctional in diabetic conditions[18] and they claim in that CD14+ cells are less affected by diabetes when compared to more primitive $CD34^+$ cells[39]. The group suggests that $CD14^+$ cells could provide a therapeutic option for people with diabetes, as these cells improve wound healing and vascular growth. This thesis shows that $CD14^+$ cells are definitely affected in patients with diabetes and that autologous cells could only be used for transplantation if these progenitors could somehow be converted from pro-inflammatory phenotype to a pro-angiogenic phenotype.

8.2 Future directions

A couple of suggestions for future directions have already been made including better characterization of true EPC and their progenitors. Clonal assays will be needed to answer questions about precursor subsets. The proposed mouse model (chapter 5) could help exploring these fields. Another interesting concept is skew of progenitor cells under adverse metabolic conditions. It would be very interesting to see if this observation can be confirmed in patients?
Recently microRNAs have been implicated in the regulation of hematopoietic lineage development[40]. MicroRNAs might be candidates to direct EPC and macrophage differentiation. It would be very interesting to elucidate whether microRNAs are indeed involved in these processes and to see if these microRNAs are altered under hyperglycemic conditions. If so, microRNA profiling of circulating progenitor populations could provide a potent means to monitor the susceptibility of patients to hemodynamic and metabolic risk

factors. Finally, future research on the reversal of EPC dysfunction needs to be directed towards interventions that convert pro-inflammatory phenotypes towards pro-angiogenic phenotypes.

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Chapter 9

Summary / Samenvatting

Cindy J.M. Loomans

English Summary

Endothelial Progenitor Cells (EPC) are bone-marrow derived cells that play important roles in several vascular processes. Progenitor cells in the bone marrow can be triggered to expand and to mobilize into the periphery through a cascade of stimulatory signals released by damaged or ischemic tissue. Homing of these progenitor cells towards injured or hypoxic areas can facilitate necessary processes like angiogenesis and vasculogenesis at the site.

Postnatally, EPC are needed to maintain the integrity of the endothelium (re-endothelialization) and to augment wound healing or to vascularize hypoxic areas (neovascularization). Complex networks of different signals and regulators have been identified to be involved in these processes, but the exact mechanisms are not completely understood. Unraveling these complex systems, however, could be very beneficial for the treatment of vascular diseases.

In this thesis, we focus on different aspects of EPC biology. We look at the nature of these progenitor cells in a normal environment, but we also look at the possible role of EPC in the pathogenesis of vascular disease in patients with diabetes mellitus.

In chapter 5, a transgenic mouse model expressing a green fluorescent protein under the control of an endothelial specific promoter is introduced, in which the origin and function of BM-derived EPC could be further explored. Using this transgenic mouse model, cells with endothelial-lineage commitment could be distinguished from other closely related myeloid cells like Macrophages (Mph) and Dendritic cells (DC). We show that short-term cultured EPC are mainly derived from a specific myeloid precursor fraction in the BM, with an abundant expression of both CD31 and Ly6C proteins. In chapter 2 we look at more basic functional properties of different subsets of EPC and we found that in an *in vitro* angiogenesis model, EPC did home in to sites of neovasculogenesis and also proliferate at these sites. Furthermore, part of these EC progenitors did differentiate into Endothelial Cells and they were capable of incorporating into the newly formed vessel structures. Moreover, EPC were able to enhance neovasculogenesis.

Data presented in chapter 5 and 7 suggest a new role for EPC as they may also function as cells involved in immune surveillance. Endothelial function can be severely impaired and ischemic vascular events induced in the diabetic state. Therefore, we investigated if EPC could somehow play a role in the pathophysiology of vascular disease in patients with

diabetes. In Chapter 3, we indeed observed a 40% reduction in the circulating number of short-term cultured EPC in patients with type 1 Diabetes. This finding was directly related to the severity of the disease as we demonstrated a significant inverse correlation between the number of circulating EPC and glycemic control (HbA1C %). Similar results were also found in a hyperglycemic mouse model (chapter 6), further supporting the hypothesis that hyperglycemia indeed does affect EPC numbers.

Chapter 6 describes a mechanism possibly involved in this lower number of EPC found in hyperglycemic conditions, namely altered differentiation of myeloid precursor cells. Data show that differentiation of myeloid progenitors from BM derived from hyperglycemic mice is more directed towards Mph and that this happens at the cost of the generation of EPC. In addition to a reduced number of EPC due to hyperglycemia, chapter 3 and 6 also describe an impaired angiogenic capacity of the EPC derived from hyperglycemic subjects.

Chapter 3 describes how processes like oxidative stress, known to be induced by high glucose levels in EC, could be involved in this so-called EPC dysfunction. Data from chapter 6 show that the dysfunctional EPC also display higher proinflammatory capacities, which is a concern as they may contribute to an adverse (immune) response that may be pro-atherogenic, contributing to progressive ischemic vascular disease.

Chapter 6 and 7 suggest possible mechanisms and drug treatments to (partially) overcome the effects of hyperglycemia on EPC by intervention with either a statin (Chapter 6, *in vitro*) or with a PPARγ agonist (chapter 7, *in vivo*).

Adding atorvastatin *in vitro* to BM differentiation cultures increases the number of EPC and conversely decreases the number of macrophages. In addition, the altered angiogenic properties of the EPC derived from the hyperglycemic animals could be recovered with atorvastatin use. Our observation that statins can counteract these unfavorable effects, may not only provide a helpful tool to elucidate the molecular mechanism underlying EPC dysfunction but may also contribute to new treatment strategies or even prevention strategies for vascular disease in patients with diabetes. In Chapter 7, transcriptional profiles of myeloid cells, committed to the monocytic lineage, as well as EPC were analyzed from type 2 diabetes patients treated for four weeks with the PPARγ agonist pioglitazone and compared with patients who received placebo. A marked overall transcriptional repression was observed in differentially expressed genes of both monocytes (83.3%) and EPC (91.9%). This study provides *in vivo* evidence of transcriptional transrepression and the potent anti-inflammatory properties of pioglitazone in myeloid cells in diabetes and might point to a new therapeutic mode of action of thiazolidinediones.

Nederlandstalige Samenvatting

Endotheel Progenitor Cellen (EPC) zijn in het bloed circulerende cellen die uit het beenmerg afkomstig zijn. Zij kunnen niet alleen een belangrijke bijdrage leveren aan het onderhoud van het endotheel maar ze kunnen ook bijdragen aan de vorming van nieuwe bloedvaten. Onder invloed van verschillende prikkels kunnen de voorlopercellen, of stamcellen, aangezet worden tot celdeling en kunnen deze vanuit het beenmerg mobiliseren naar het perifeer bloed. In de bloedbaan kunnen de EPC gerekruteerd worden naar de beschadigde vaatwand of naar de plaats waar nieuwe vaatjes gevormd moeten worden, vaak ischemisch weefsel. Daar kunnen de EPC zelf tot endotheel cel differentiëren of angiogene processen aansturen om nieuwe vaatjes te vormen of ze kunnen beschadigd endotheel vervangen door nieuw endotheel. Bij bovengenoemde processen zijn vele factoren betrokken en het is niet bekend hoe EPC precies werken. Het ontrafelen van deze processen kan een doorbraak betekenen op het gebied van hart- en vaatziekten, waarbij het endotheel van de patiënten voortdurend onder hoge druk staat door metabole veranderingen en deze EPC dus van groot belang zouden kunnen zijn.

In dit proefschrift beschrijven we verschillende aspecten van de EPC biologie. Enerzijds kijken we naar het gedrag van EPC in een normale omgeving, maar we gaan ook uitgebreid in op een mogelijke rol voor EPC in het vaatlijden van diabetes patiënten. In hoofdstuk 5 introduceren we een transgeen muizen model waarin de muizen een groen fluorescerend eiwit produceren onder de controle van een endotheel specifieke promotor. In dit transgeen muizen model kunnen we de oorsprong van de EPC en de functie van de EPC bekijken en kunnen we de EPC gemakkelijk onderscheiden van gerelateerde andere myeloide cellen als macrofagen en dendritische cellen. We laten zien dat EPC afkomstig uit het beenmerg voor een groot gedeelte ontstaan uit een specifieke myeloide voorloper fractie van het beenmerg met een hoge expressie van zowel CD31 als Ly6C eiwitten. Meer functionele eigenschappen van EPC worden besproken in hoofdstuk 2, waar zowel homing van EPC als de proliferatie op plaatsten waar vaat-nieuwvorming plaatsvindt worden beschreven in een *in vitro* angiogenese model. Ook wordt beschreven dat een gedeelte van de EPC kan differentiëren tot EC, dat deze kunnen incorporeren in een monolaag van volwassen endotheel cellen en dat ze mate van vaatvorming kunnen stimuleren. Een potentiële nieuwe rol voor EPC in het immuunsysteem wordt beschreven in hoofdstukken 5 en 7. Diabetische condities, waaronder hoge bloedsuikers, kunnen endotheel disfunctie veroorzaken en diabetes mellitus is geassocieerd met verhoogde kans op hart- en vaatziekten. Daarom

hebben wij onderzocht of EPC een rol spelen in de ontwikkeling van vaatlijden in deze patiënten. Hoofdstuk 3 laat zien dat type 1 diabetes patiënten een sterk verminderd (40%) aantal circulerende EPC hebben. De reductie in het aantal cellen was negatief gerelateerd aan de mate van bloedglucose instelling van de patiënten. Het concept dat verhoogde glucose bloedspiegels het aantal EPC kan beïnvloeden wordt verder gesteund door observaties in hoofdstuk 6, waar in een hyperglycemisch muizen model ook een verminderd aantal EPC wordt gevonden eveneens negatief gerelateerd is aan de HbA1c percentages van de muizen. Een mechanisme mogelijk verantwoordelijk voor het verminderde aantal EPC is een veranderde differentiatie van myeloide precursors onder hyperglycemische condities (hoofdstuk 6). We laten zien dat er een verschuiving is in de differentiatie van myeloide precursors naar macrofagen ten koste van de EPC. Naast een verminderd aantal EPC vinden we ook een verstoorde angiogene capaciteit van de EPC van zowel type 1 diabetes mellitus patiënten (hoofdstuk 2) als die van hyperglycemische muizen (hoofdstuk 6). Daarnaast beschrijft hoofdstuk 6 ook dat deze disfunctionele EPC hogere pro-inflammatoire eigenschappen hebben. Dit zou *in vivo* een ongunstige ontstekingsreactie kunnen opleveren op de plaats van een endotheel beschadiging, bijdragend aan de progressie van atherogene processen in de vaatwand.

Hoofdstuk 3 beschrijft een mogelijk ander mechanisme achter EPC disfunctie, namelijk oxidatieve stress. Hoge glucose kan zorgen voor oxidatieve stress in endotheel cellen en mRNA profielen laten zien dat deze mechanismen stress en disfunctie zouden kunnen veroorzaken in EPC.

Nu dat we weten dat EPC disfunctie mogelijk een rol zou kunnen spelen in de pathofysiologie van vaatlijden in diabetes patiënten is het de vraag of we deze disfunctie kunnen overwinnen met behulp van bestaande therapeutische middelen. Hoofdstuk 6 laat zien dat *in vitro* toevoeging van de HMG-CoA reductase remmer atorvastatine, het aantal EPC verhoogd en tegelijkertijd het aantal macrofagen kan verminderen. Daarnaast heeft atorvastatine ook een positieve invloed op de angiogene capaciteiten van EPC. Deze observaties, dat statines een positieve invloed hebben op het aantal EPC en tevens ook op de functie, zorgt voor meer inzicht in de pathofysiologie achter EPC disfunctie en kan (mits het dezelfde effecten laat zien *in vivo*) invloed hebben op betere behandelingsstrategieën voor diabetes patiënten met vascularaire complicaties of zelfs voor preventie van hun vaatlijden.

In hoofdstuk 7 worden effecten van een andere therapeutisch middel pioglitazone besproken. Pioglitazone is een PPARγ agonist en wordt gebruikt voor verbetering van

insulinegevoeligheid van type 2 diabetes mellitus patiënten. In een gerandomiseerde, crossover, placebo-gecontroleerde, dubbel blinde studie van type 2 diabetes mellitus patiënten die voor korte tijd behandeld werden met pioglitazone, zijn transcriptionele mRNA profielen geanalyseerd van zowel monocyten als EPC. Een duidelijke algemene transcriptionele onderdrukking werd waargenomen in de differentieel gereguleerde genen van zowel monocyten (83,3%) als EPC (91,9%). Naast de algemene transcriptionele transrepressie zagen we een down-regulatie van pro-inflammatoire genen in zowel monocyten als EPC door pioglitazone behandeling, wat therapeutisch een nieuwe weg zou kunnen inleiden om progressie van atherogene complicaties in diabetes patiënten tegen te gaan.

List of abbreviations:

ACE	Angiotensin I-Converting Enzyme
acLDL	acetylated Low-Density Lipoproteins
AGE	Advanced Glycation Endproducts
Ang	Angiotensin
AU	Arbitrary Units
bFGF	basic Fibroblast Growth Factor
BMI	Body Mass Index
BM-MNC	Bone marrow Mononuclear Cells
BP	Blood Pressure
BS-1	Bandeiraea Simplicifolia -1
CAC	Circulating Angiogenic Cells
CAD	Coronary Artery Disease
CB	Cord Blood
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CEP	Circulating Endothelial Progenitor Cells
CFU-EC	Colony Forming Unit –Endothelial Cells
CLC	Cobble Stone-Like Cells
CM	Conditioned Media
cpm	counts per minute
CVD	Cardiovascular disease
DC	Dendritic Cells
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
EC	Endothelial Cells
ECGF	Endothelial Cell Growth Factor
ECM	Extracelllular Matrix
EGM	Endothelial Cell Growth Medium
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cells
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FFA	Free Fatty Acids
FITC	Fluorescein Isothiocyanate
Flt-1	FMS-related Tyrosine kinase 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte Colony Stimulating Factor
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GTPase	Guanosine TriPhosphatase
H_2O_2	Hydroperoxide
HbA1c	Glycosylated Hemaglobin
hCB-MNC	Human Cord Blood Mononuclear Cells
HIF	Hypoxia Inducible Factor
HLA	Human Leucocyte Antigen
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HSC	Hematopoietic Stem Cell
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intra Cellular Adhesion Molecule
IL	Interleukin
IP	Intraperitoneal
IRF4	Interferon Regulatory Factor 4

KDR	Kinase insert Domain Receptor
LNGFR	Low-Affinity Nerve Growth Factor Receptor
LPS	Lipopolysaccharide
Ly6c	Lymphocyte antigen 6c
MACS	Magnetic Affinity Cell Sorting
MCP-1	Monocytes Chemo attractant Protein-1
M-CSF	Macrophage Colony Stimulating Factor
MECA	Mouse Endothelial Cell Antigen
MH	Murohara Endothelial Cell Growth Medium
MHC	major histocompatibility complex
MLR	Mixed Lymphocyte Reaction
MMP	Matrix Metalloproteinase
MNC	Mononuclear cells
MnSOD	Magnesium Superoxide dismutase
Mph	Macrophages
MVEC	Microvascular Endothelial Cells
NADPH	reduced form of Nicotinamide Adenine Dinucleotide Phosphate
NFκB	Nuclear factor κ
NO	Nitric oxide
PB-MNC	Peripheral Blood Mononuclear Cells
PDGF	Platelet-derived Growth Factor
PE	Phycoerythrin
PI3	Proteinase Inhibitor 3
Pio	Pioglitazone
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PPAR	Peroxisome Proliferator-Activated Receptors
RAAS	Renin-Angiotensin A
RAGE	Receptor for Advanced Glycation Endproducts
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCA-1	Stem Cell Antigen 1
SD	Standard Deviation
SDF	Stromal cell-Derived Factor
SEM	Standard Error of Mean
SMC	Smooth Muscle Cell
SP	Side Population
STZ	Streptozotocin
tg	transgenic
Th1	T helper cell 1
TNFα	Tumor Necrosis Factor Alpha
UEA	Ulex europaeus agglutinin
VCAM	Vascular Cell Adhesion Molecule
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
vWF	von Willebrand factor
wt	wildtype

Curriculum Vitae

Cindy Johanna Maria werd geboren op 2 juni 1976 te Bergeijk. Na het behalen van haar HAVO diploma op het Hertog Jan College in 1993, begon zij haar opleiding tot Biomedisch analist aan de Hogeschool Utrecht. Nadat ze haar propedeuse jaar in Utrecht had behaald heeft ze haar hoger beroepsopleiding voortgezet aan de Fontys Hogeschool Eindhoven waar ze haar diploma in 1997 in ontvangst kon nemen.
Daarna heeft ze voor anderhalf jaar als analiste gewerkt aan het Nederlands Kankerinstituut op de afdeling Moleculaire Carcinogenese. In 1999 besloot ze voor twee jaar naar Amerika te gaan om daar als analiste te werken aan de Universiteit van California in San Diego (UCSD) op de afdeling Immunologie. In 2001 werd zij aangesteld als assistent in opleiding (AIO) aan de universiteit van Utrecht op de afdeling vasculaire geneeskunde in het Academisch Ziekenhuis Utrecht. Na anderhalf jaar heeft ze haar promotor gevolgd in een verhuizing richting het Leids Universitair Medisch Centrum (LUMC) en is ze daar aangesteld als AIO bij de afdeling Nierziekten voor de resterende 3 jaar. In deze tijd heeft ze ook veel onderzoek verricht in het lab van dr. Staal aan de Erasmus Universiteit in Rotterdam. De resultaten van 4,5 jaar onderzoek zijn beschreven in dit proefschrift.

Na het afronden van haar AIO onderzoek in Leiden is Cindy begin 2006 weer richting Amerika gegaan om een gedeelte van haar eerste post-doc baan te werken in het Joslin Diabetes Center, geaffilieerd met de Harvard Universiteit in Boston. Dit onderzoek wordt na anderhalf jaar voortgezet in het LUMC in Leiden en is gericht op de transplantatie van eilandjes van Langerhans in diabetes patiënten.

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