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Vasculogenesis in Diabetes-Associated Diseases: Unraveling the Diabetic Paradox

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1. Introduction

During several decades it was thought that in the adult, vascular growth and remodelling was exclusively dependent on the activation of angiogenesis, being the process of vasculogenesis restricted to embryonic life (Risau & Flamme, 1995; Risau, 1997). This long-lasting belief has come to an end in the late nineties, with the isolation from adult peripheral blood (PB) of angioblast-like circulating Endothelial Progenitor Cells (EPCs) (Asahara et al., 1997). The discovery of bone marrow (BM)-derived EPCs with angioblastic morphological and functional properties was a landmark in vascular biology that forever has changed the concept of neovascularization. Numerous studies have demonstrated that EPCs residing in the BM could be mobilized to the peripheral circulation, migrate to neoangiogenic sites and partake *in vivo* in the pathophysiological development of vascular networks, by differentiating into functional, mature endothelial cells (ECs) (Asahara et al., 1999a, 1999b; Goon et al., 2007; Grant et al., 2002; Lyden et al., 2001; Takahashi et al., 1999). However, much controversy has accompanied this field over time, particularly regarding the phenotypic characterization of EPCs. In fact, this cell population does not have specific cell surface markers, sharing a diversity of membrane receptors with other BM-derived cells (Hristov et al., 2003; Peichev et al., 2000). Additionally, several subsets of EPCs have been identified and together with other lineages of precursor cells were found to be differentially recruited to neovascular foci contributing synergistically to vasculogenic neof ormation (Gulati et al., 2003; Hur et al., 2004; Lyden et al., 2001; Yoder et al., 2007; Yoon et al., 2005a). Since their identification, an increasing body of evidence has definitely revealed the important properties and roles played by EPCs in several vascular-related diseases, such as peripheral vascular disease (Asahara et al., 1999a, 1999b; Takahashi et al., 1999), tumor neovascularisation (Asahara et al., 1999a, 1999b; Lyden et al., 2001) and vascular complications associated to diabetes (Goon et al., 2007; Grant et al., 2002; Egan et al., 2008; Fadini et al., 2005). The metabolic alterations present in diabetic individuals are known to profoundly affect vascular biology, being responsible for the impairment of macro- and microvascular beds (Fadini et al., 2006a; Werner et al., 2005). Diabetes associated vascular complications involve distinct modifications in neovascular formation, which is reduced in ischemic heart and limbs and increased in the retina, defining the diabetic paradox (Ciulla et al., 2003). The vasculogenic process seems to play a central dual role in this paradoxal puzzle: systemically, diabetes-associated hyperglycemia, insulin resistance, hypertension and oxidative stress, can simultaneously

injure the endothelium and deleteriously affect EPCs biological functions, impairing efficient systemic vascular repair and promoting peripheral vasculopathy (Povsic & Goldschmidt-Clermont, 2008); locally in the retina, the specific microenvironment may contribute to the local recruitment of EPCs, promoting increased vessel growth (Goon et al., 2007; Grant et al., 2002). Although still under investigation, these divergent features seem related to a differential regulation of neovascular mechanisms, which responde differently to ischemia-induced depletion of angiogenic factors in systemic conditions, or to the local induction of vascular growth factors in the diabetic retina (Duh & Aiello, 1999). Due to the growing importance of vasculogenesis in diabetic vascular complications, interventions differentially modulating EPCs levels/functions may be regarded as therapeutic strategies. Systemically, vasculoprotective agents provided with beneficial cardiovascular effects, have showed to improve endothelium-dependent vascular function, by restoring EPCs properties and actions (Liang et al., 2009). Local delivery of anti-angiogenic drugs to the retina may have positive effects by hampering EPCs recruitment in proliferative diabetic retinopathy (PDR) (Chung et al., 2011). However, one might not neglect that diabetic patients frequently display both neovascular peripheral decrease and retinal increase of vessel growth. So, important questions are raised regarding the therapeutic use of EPCs in the promotion of peripheral vascularization and the impairment of EPCs-induced retinal neovascular formation and increased ischemia. Several issues need to be addressed in order to optimize the therapeutic use of EPCs and to get further information on the putative harmful side effects. Future research is mandatory in order to elucidate the complex molecular mechanisms governing the diabetic-vasculogenic paradigm, bringing novel insights into the safety on the therapeutic modulation of EPCs in diabetes vascular disorders.

2. Endothelial progenitor cells (EPCs) and postnatal vasculogenesis

Vasculogenesis and angiogenesis are the fundamental processes by which new blood vessels are formed. Vasculogenesis is defined as the differentiation of EPCs or angioblasts into ECs and the *de novo* formation of a primitive vascular network, whereas angiogenesis is the growth of new capillaries from pre-existing blood vessels (Risau & Flamme, 1995; Risau, 1997). In the embryo, blood vessels form through both vasculogenesis and angiogenesis. Vasculogenesis occurs during early embryonic development and mediates the *de novo* vessel formation from angioblasts of mesodermal origin, which differentiate into mature ECs assembling into a primary capillary plexus (Risau & Flamme, 1995). Subsequently, this primitive vascular network expands by angiogenesis, where new blood vessels arise from the proliferation and migration of the pre-existing ECs (Folkman, 1984; Risau, 1997). During several decades it was thought that in the adult, vascular growth and remodelling was exclusively dependent on the activation of angiogenesis, and that the process of vasculogenesis was restricted to embryonic life. This prevailing dogma has come to an end over a decade ago, with the identification of circulating BM-derived EPCs in 1997 by Asahara and collaborators, which have isolated a population of angioblast-like CD34+ circulating EPCs from adult PB. In *in vitro* cultures, these cells presented an increased proliferation rate and exhibited endothelial morphological and functional properties (Asahara et al., 1997). However, this pioneering work was criticized and the true identity of the putative EPCs was questioned. In fact, besides the functional characteristics of these progenitor cells, namely their high proliferative capacity, this report did not provide any additional information regarding other specific functional characteristics. Additionally, in

Asahara's work the CD34 antigen was used to select EPCs, however the authors did not present a defined set of other phenotypic markers that could unambiguously identify this cell population allowing their sole isolation. In fact, CD34 is also expressed in sub-groups of hematopoietic stem/progenitor cells and mature ECs and does not specifically discriminate EPCs. Nevertheless, the work of Asahara *et al.* set a landmark in the field of vascular biology, for being the first to suggest that vasculogenesis could occur during adult life. Besides the controversy, after this initial report, a boom of novel studies corroborated Asahara's hypothesis, presenting novel evidence for the existence of postnatal EPCs and their functional role in pathophysiological neovascular processes. Several reports demonstrated that a population of EPCs residing in the BM could be mobilized to the peripheral circulation, migrate to neoangiogenic foci and partake *in vivo* in the development of vascular networks, by differentiating into functional matured ECs and incorporating the vasculature (Asahara *et al.*, 1999a, 1999b; Lyden *et al.*, 2001; Shi *et al.*, 1998). EPCs mobilization from the BM and homing to neovascular foci occurred in response of progenitor cells to specific angiogenic stimuli. The initial steps in mobilization involved the activation of matrix metalloproteinase (MMP)-9, which catalyses the conversion of membrane-bound Kit ligand to soluble Kit ligand. The subsequent cKit-positive progenitor cells are disengaged and can then move from the osteoblastic to the vascular zone of the BM. This process is enhanced by elevated levels of the chemokines Stromal Derived Factor (SDF)-1 and Vascular Endothelial Growth Factor (VEGF) (Heissig *et al.*, 2002) and appears to be endothelial Nitric Oxide (eNO) dependent (Aicher *et al.*, 2003). Increased eNO levels stimulate the passage of EPCs through BM sinusoidal endothelium and their entrance in the blood stream, where they are further recruited to neoangiogenic foci (Aicher *et al.*, 2003, 2005).

3. EPCs phenotypic and functional properties

Since their identification, the phenotypic characterization of EPCs has emerged a major setback in the field. Their isolation, identification and characterization were mostly hampered by the lack of EPCs-specific surface markers. Over time some consensus has apparently been reached and EPCs were considered to be the cell population characterized by the concomitant expression of: the early hematopoietic stem cell markers CD34 and CD133 (former AC133) and VEGF receptor-2 (VEGFR-2) (Hristov *et al.*, 2003; Peichev *et al.*, 2000). However, it has been suggested that human CD34+CD133+VEGFR2+ cells comprise a population of cells that are not only EPCs, but distinct primitive hematopoietic progenitors, which also express markers such as CD45 and are devoid of vessel formation capabilities (Case *et al.*, 2007; Timmermans *et al.*, 2007). Other findings have demonstrated the existence of novel subsets of progenitor cells with an involvement in vascular repair. It was reported the presence in PB of a stem cell population which lack the CD34 antigen and is able to differentiate into CD34+CD133+ EPCs, and acquire a more mature endothelial phenotype (Friedrich *et al.*, 2006). Additionally, other populations with endothelial repair capabilities which express additional cell surface markers, including the receptor for SDF-1, the chemokine (C-X-C motif) receptor (CXCR)-4 have also been identified (Egan *et al.*, 2008). The characterization of these different subpopulations of EPCs is due to the different methodology used for cell isolation. To date, there are three main techniques that have been used to select, identify and characterize EPCs (Hirschi *et al.*, 2008; Yoder, 2009). The cell types isolated using the different protocols are not phenotypically similar and as such, their

potential to influence neovascularization and/or vascular repair may vary. Consequently, this may offer an explanation as to the differences observed amongst similar studies which presented divergent results.

3.1 Methods for EPCs isolation and characterization

3.1.1 Culture of isolated mononuclear cells

This method involves the isolation of mononuclear cells from PB or BM using density gradient centrifugation and the plating of these cells on fibronectin-coated substrates and cultured in medium supplemented with endothelial growth factors. Approximately 3 days later, non-adherent cells are removed from the culture and fresh media is added to the remaining cells which continue to be cultured and further analysed. These spindle shaped-adherent cells express EC markers and functional qualities, such as endocytosis and acetylated low-density lipoprotein (LDL) uptake. However, unlike progenitor cells they display low proliferative capacity, cannot form EC tube-like structures in an *in vitro* angiogenesis assay model, but do display panleukocyte and monocytic/macrophage markers, such as CD14 and CD45 (Rehman et al., 2003; Zhang et al., 2006, 2007). In support of this finding, previous data showed that monocytes have high affinity for fibronectin, and that 90% of cells from PB samples which adhere to coated dishes are of monocytic origin (Freundlich & Avdalovic, 1983). Moreover, a recent study by Prokopi *et al.* suggested how these putative EPCs may acquire EC markers. According to their data, the method used for mononuclear cell isolation from PB leads to the contamination of cells with platelets, which also express endothelial markers such as CD31 and von Willebrand factor (vWF) (Prokopi et al., 2009). During a 7 day mononuclear cell culture, the platelets are degraded into microparticles, vesicles which retain specific antigens from the cell of origin. So, adherent day 1 mononuclear cells presented as CD31 negative, but by day 7 expressed the CD31 antigen, along with platelet-specific markers, following the uptake of degraded platelet microparticles and a transfer of cell antigens. Additionally, depletion of platelet microparticles from the EPCs culture media also removed the angiogenic properties often attributed to EPC culture medium, indicating that this *in vitro* property may be dependent on initial platelet presence following mononuclear cell isolation (Kirton & Xu, 2010; Rehman et al., 2003; Urbich et al., 2005).

3.1.2 Fluorescence activated cell sorting (FACS) and *in vitro* culture

One of the most currently used methods for EPCs separation, involves cell labelling with antigen specific antibodies and FACS analysis. This was the technique used by Asahara *et al.* in their pioneering work to isolate CD34+ mononuclear cells (Asahara et al., 1997). Since as aforementioned, CD34 does not selectively discriminate EPCs, following studies also included the use of CD133 combined with CD34 and VEGFR-2 (Peichev et al., 2000), to ensure that only progenitor cells were isolated as apposed to circulating ECs that have detached from the vessel wall (Blann & Pretorius, 2006; Ingram et al., 2005). These cells were cultured for 2 days and non-adherent cells re-plated for further 14 days, giving similar clustered colonies. Upon differentiating into mature ECs, EPCs lose the expression of CD133 and start exhibiting classical EC morphology and features, such as the expression of the endothelial markers vWF and vascular endothelial cadherin (VE-cadherin) and the capacity to uptake acetylated LDL (Peichev et al., 2000; Shi et al., 1998). Several subsequent studies successfully isolated this cell type from adult PB, umbilical cord blood and fetal liver, using

a combination of these three markers (Timmermans et al., 2009). However, further controversy has arisen since, it was reported that CD34+CD133+VEGFR2+ cells comprise a mixed population of EPCs and primitive hematopoietic progenitors, which express also CD45 and also lack the ability to form vessel-like structures (Case et al., 2007; Timmermans et al., 2007).

3.1.3 *In vitro* colony forming cell assays

Using this technique independent groups have shown that in adult PB mononuclear cells there are two distinct EPCs populations, which form *in vitro* Early Outgrowth Colonies (EOCs) and Late Outgrowth Colonies (LOCs) (Gulati et al., 2003; Hur et al., 2004). Additional studies have used different designations for these cell types: EOCs were also named early EPCs (eEPCs) and LOCs are also known as Outgrowth Endothelial Cells (OECs) (Medina et al., 2010, Yoon et al., 2005a) Although the biology of these endothelial progenitor-like cells is still under investigation, they seem to present different phenotypes, surface antigens and display diverse vasculogenic features *in vitro*. EOCs and LOCs are primarily characterized based on their morphology and chronology of appearance following *in vitro* culture. EOCs appear in culture within 7 days, emanating from a central cluster of cells, exhibiting spindle-shaped morphology and having a peak growth at 2-3 weeks after which they cannot be further expanded (Gulati et al., 2003; Hur et al., 2004). LOCs generally appear after 3 weeks and exhibit a "classic endothelial" phenotype, having an increased expansion potential. LOCs seem more capable of *in vitro* morphogenesis into capillary tubes, the best approximate true definition of an EPC, a competent progenitor cell whose terminally differentiated progeny are mature ECs (Gulati et al., 2003; Hur et al., 2004; Yoder et al., 2007). This capillary-forming capacity is minimal or nonexistent within EOCs, which are thought to have a paracrine role by supporting LOCs differentiation and capillary formation, through the release of pro-angiogenic molecules and by inducing the activation of MMPs (Gulati et al., 2003; Hur et al., 2004; Yoon et al., 2005a). Phenotypically, EOCs additionally express the monocyte/macrophage marker CD14, which is absent among mature LOCs, and both populations may concomitantly express CD34 and VEGFR-2 (Yoon et al., 2005a). However, besides the reported alterations in phenotypic markers and the dissimilar biological properties, there was a lack of information regarding molecular differences between EOCs and LOCs. Recently, a study has provided a detailed molecular fingerprint of these two EPC subtypes, designated in this report by eEPCs and OECs. Medina and collaborators have shown that eEPCs and OECs have strikingly different gene/protein expression signatures (Medina et al., 2010). As evaluated by microarrays, many highly expressed transcripts in eEPCs were hematopoietic specific, including the Runt-related transcription factor (RUNX1) and the protein tyrosine kinase LYN; and with links to immunity and inflammation (Toll-Like Receptors, TLRs; CD14; Human Leukocyte Antigens, HLAs). On the other hand, OECs presented several highly expressed transcripts involved in vascular development and angiogenesis-related signaling pathways, such as the receptor tyrosine kinase Tie2, eNOS and Ephrins. Similarly, proteomic analysis revealed that 90% of spots identified by 2D gel electrophoresis analysis were common between OECs and endothelial cells while eEPCs shared 77% with monocytes. This study provided evidence that eEPCs are hematopoietic cells with a molecular phenotype linked to monocytes; whereas OECs exhibit commitment to the endothelial lineage, corroborating at the molecular level all the previous studies that have phenotypically

characterized these cell populations. These findings indicate that OECs are the subtype with vasculogenic capability and that functionally integrates neovascular foci, and it should be an attractive cell candidate for inducing therapeutic angiogenesis.

Overall, these reports indicate that EPCs represent a heterogeneous population of cells, some of monocytic nature and others with a pro-angiogenic potential. Depending on the study model, cell isolation method and cell subtype used, the pro-angiogenic effects are a consequence of the direct vascular integration, the paracrine release of growth factors and cytokines, or the complex interactions with other cellular components like monocytes or platelets. Nonetheless, most of the reports involving *in vitro* and *in vivo* EPCs studies do not usually make a distinction between EOCs (eEPCs) and LOCs (OECs), analyzing both populations as a whole. As aforementioned, this may explain divergences observed amongst similar studies.

4. Vasculogenesis and *in vivo* neovascular formation in pathophysiological processes

Studies carried on experimental models have shown that postnatal vasculogenesis could take place under certain physiological and pathological settings. Further, it was also suggested that other BM-derived hematopoietic stem/myeloid progenitor cells, named as accessory cells, could be co-recruited to neoangiogenesis foci, and support vascular growth in a paracrine fashion through the release of pro-angiogenic factors or by contributing to extracellular matrix remodeling (De Palma et al., 2005; Fang & Salven, 2011; Grunewald et al., 2006; Kaplan et al., 2005; Lyden et al., 2001; Takakura, 2006). Due to the relevant role of neovascularization for tumor growth and metastization, several pre-clinical studies evaluated EPCs functions and their contribution for malignant development (Asahara et al., 1999a, 1999b; Lyden et al., 2001). Additionally and corroborating experimental data, it was also reported that a percentage of BM-derived EPCs can integrate human tumour-associated neovasculature (Peters et al., 2005). Nonetheless, despite all these evidence Purhonen et al. have suggested that BM-derived circulating EPCs do not contribute to vascular endothelium and are not needed for tumor development, raising novel controversy in the field (Purhonen et al., 2008). Besides tumor neovascular formation, further studies confirmed that EPCs play also a role in: vascular homeostasis and repair (Asahara et al., 1999a; Kirton & Xu, 2010; Shantsila et al., 2007), wound healing (Asahara et al., 1999b), bone regeneration (Matsumoto et al. 2008), myocardial infarction (Porto et al., 2011; Shintani et al., 2001), limb ischemia (Asahara et al., 1999a, 1999b; Takahashi et al., 1999), burn individuals and escharectomy (Foresta et al., 2011; Gill et al., 2001) and vascular complications associated to diabetes (Goon et al., 2007; Grant et al., 2002; Egan et al., 2008; Fadini et al., 2005).

5. Vasculogenesis and the diabetic paradox

Diabetes mellitus (DM) is a common costly chronic disease and its incidence is rapidly increasing worldwide. Once considered primarily as a risk factor for heart disease, diabetes has now become a high profile public health concern in its own right, due to the escalating epidemic of diabetes in older people, and the emergence of type 2 DM (T2DM) in children. In fact, individuals with T2DM account for most of this augmentation in the general population. An important part of this rise is attributed to changing living conditions, including overweight and obesity, sedentary behaviour, and unhealthy lifestyle (Perkins,

2004; Zimmet et al., 2001). Vascular complications in T2DM are a significant cause of human morbidity and mortality, by affecting multiple organs, in particular the cardiovascular system, through the promotion of atherosclerosis (Haffner et al., 1998; Nakagami et al., 2005; Werner et al., 2005). Diabetic vascular alterations in different organs occur by distinct modifications in neovascular formation, which is decreased in systemic cardiovascular disease (CVD) and increased in DR. This diabetic paradox has been attributed to the differential regulation of neovascular mechanisms, which responde differently to ischemia in diabetic conditions (Duh & Aiello, 1999). The vasculogenic process seems to play a central dual role in this paradoxal puzzle: by one hand diabetes-associated hyperglycemia, insulin resistance, hypertension and oxidative stress, can simultaneoulsy injure the endothelium and deliteriously affect EPCs functions, thus preventing efficient systemic vascular repair, favoring the development of peripheral vasculopathy (Povsic & Goldschmidt-Clermont, 2008); on the other hand the specific retinal *millieu* may promote the local recruitment of EPCs, contributing to increased vessel growth in PDR (Goon et al., 2007; Grant et al., 2002). Although the complex mechanisms governing this diabetic-vasculogenic paradox are still under investigation, novel evidence links alterations in EPCs biological functions to diabetic vascular complications, and will be further discussed.

6. Diabetic vasculopathy and altered vasculogenesis

Diabetes is characterized by a systemic pro-inflammatory state and generalized endothelial dysfunction (EDys). EDys ultimately represents the unbalance between endothelium injury and the endogenous capacity for endothelial repair (Costa et al., 2007). Compelling evidence suggested that hyperglycemia, insulin resistance, hypertension and oxidative stress, simultaneously promote endothelial damage and deliteriously affect EPCs functions, thus preventing efficient vascular repair and favoring the development of atherosclerotic lesions (Madonna & De Caterina, 2011; Povsic & Goldschmidt-Clermont, 2008). Amongst all these risk factors, increased glycemic levels and excessive oxidative stress seem to be the major causal factors underlying both endothelial injury and vasculogenic impairment (Callaghan et al., 2005). Nonetheless, the complex interplay between all the aforementioned conditions is thought to sinergistically decrease endothelial regeneration by altering EPCs biological activities, such as: reducing EPCs migration (Kränkel et al., 2005; Vasa et al., 2001); impairing their mobilization (Fadini et al., 2006b; Gallagher et al., 2007; Kang et al., 2009; Yao et al., 2006); promoting premature senescence (Higashi et al., 2002); inhibiting integrative and morphogenic capacities (defective adhesion, colony-forming ability and tubulization) (Fadini et al., 2006a; Tepper et al., 2002) and inducing EPCs apoptosis (Chen et al., 2010; Shen et al., 2010). These alterations on EPCs features have been associated with deficient endogenous re-endothelialization/neovascularization, being a potential indicator of diabetic CVD severity (Egan et al., 2008; Fadini et al., 2005; 2006b). In conjunction with deficient EPCs functions, the production of chemotactic/angiogenic factors is inhibited in diabetic peripheral ischemic tissues; contributing both to poor collateral formation and insufficient perfusion. It has been shown that the expression of angiogenic factors such as, VEGF and Hypoxia-Inducible Factor (HIF)-1 α are reduced in the heart of diabetic patients during acute coronary syndromes (Marfella et al., 2004). Recently, it was also demonstrated that following the onset of acute myocardial infarction in T2DM patients, the numbers of CD133+ progenitor cells are reduced and their chemotactic responsiveness attenuated (Vöö et al., 2009). Both the defective expression of angiogenic factors and the dysfunction in EPCs

account for the delayed post-ischemic vascular healing and myocardial recovery in patients with DM. Diabetic cardiomyopathy is also characterized by an early and progressive decline in myocardial VEGF expression and reduced circulating EPCs, which contributes to diminished capillary density, decreased myocardial perfusion and impaired contractility (Yoon et al., 2005b). A significant decline in circulating EPCs was also reported in diabetic patients with peripheral arterial disease (PAD), particularly in individuals with ischemic foot lesions. EPCs levels correlated with the ankle-brachial index, the most objective diagnostic and prognostic test for lower extremity arterial disease. It was also demonstrated that decreased EPCs closely correlated with the severity of both carotid and lower limb atherosclerosis. Higher degrees of carotid stenosis, as well as, worse stages of leg claudication and ischemic lesions, were associated with lower levels of EPCs. This suggested that EPCs counts could be considered a valuable marker for atherosclerotic involvement (Fadini et al., 2005). Additionally, EPCs isolated from diabetic patients with PAD exhibited poor endothelial differentiation capacity, impaired proliferation and deficient adhesion to mature endothelium (Fadini et al., 2006a; 2006c). Recently, a study evaluated circulating levels of CD34-CD133+VEGFR-2+ EPCs in diabetic patients with CVD, revealing a decrease in this subpopulation caused by diabetes-induced apoptosis (Jung et al., 2010). Similarly, it was also proposed that a subset of CXCR-4+ progenitor cells with vascular repair capability, were decreased in the PB of T2DM patients (Egan et al., 2008). In ischemic conditions, reduced levels of CXCR-4+ precursors may respond poorly to SDF-1, preventing their efficient mobilization and recruitment from the BM niche to ischemic sites. In addition, it seems relevant to mention novel data disclosing the effects of hyperglycemia in EPCs in intrauterine life. Interestingly, the exposure to high glucose levels in a diabetic intrauterine environment was shown to diminish the clonogenic potential of neonatal EPCs, providing a new insight into the long-term cardiovascular complications observed in newborns of diabetic pregnancies (Ingram et al., 2008). Since it was reported that hyperglycemia, insulin resistance, hypertension and oxidative stress, are key factors on promoting endothelial damage and by altering EPCs functions, it will be further discussed how these conditions deleteriously affect vasculogenic events associated to peripheral vasculopathy (Madonna & De Caterina, 2011; Povsic & Goldschmidt-Clermont, 2008)

6.1 Hyperglycemia

Hyperglycemia is one of the major causal factors implicated in the development of vascular alterations (Aronson, 2008). High glucose levels are involved in the generation of advanced glycation end products (AGEs), which accumulate in the vessel wall, and by interacting with its receptors (RAGE) induce oxidative stress, increase inflammation and promote EDys (Jandeleit-Dahm & Cooper, 2008). Hyperglycemia is thought to maintain EDys conditions by directly impairing most EPCs-driven functional capabilities. Hyperglycemia was demonstrated to: decrease EPCs migration and integrative capacities (Kränkel et al., 2005); shift their differentiation into a pro-inflammatory phenotype (Loomans et al., 2009); reduce EPCs mobilization (Gallagher et al., 2007); accelerate the onset of progenitor cell senescence (Chen et al., 2007); induce apoptosis (Chen et al., 2010); inhibit EPCs colony-forming ability; decrease the number and proliferation of both early and late EPCs (EOCs and LOCs) and to impair the migration and vasculogenesis activities of LOCs, the subtype with vasculogenic-associated morphogenesis capability (Chen et al., 2007). Decreased vascular progenitor cells migration and inhibition of functional incorporation into tubular structures, were suggested to occur through

hyperglycemia-induced decrease in NO production and MMP-9 activity (Kränkel et al., 2005). Reduced mobilization of EPCs was showed to occur due to modifications in eNOS phosphorylation and activation status within the BM microenvironment, unabling efficient EPCs release from the marrow niche to the peripheral circulation (Gallagher et al., 2007; Ingram et al., 2008). Hyperglycemia-induced EPCs senescence was demonstrated to take place through multiple mechanisms such as, by promoting telomere shortening, and alterations in the p38 MAPK and NO-mediated pathways (Chen et al., 2007; Ingram et al., 2008; Kuki et al., 2006). Additionally, a novel molecular link between high glucose levels and EPCs-increased senescence has been unveiled, as it was demonstrated that the Sirtuin 1 (SIRT1) gene, which regulates cell cycle, premature senescence and apoptosis, is downregulated in EPCs. SIRT1 low expression levels impair the important cascade of intracellular events, culminating with EPCs early senescence (Balestrieri et al., 2008a). Highlighting the crucial effects of high glicemic levels, it was also reported that the number of EPCs in T2DM was significantly decreased as compared with healthy controls. Additionally, an inverse correlation between EPCs numbers, plasma glucose and glycated hemoglobin (HbA1C) was found. Further, the number and function of EPCs in patients with good glycemic control were recovered compared with those with poor glycemic control. When glucose was supplemented to *in vitro* cultures, there was a negative effect on the proliferation and viability of EPCs, in a dose-dependent manner, whereas the enhancement of apoptosis was observed (Churdchomjan et al., 2010). All these deleterious effects were reported to occur due to a direct effect of elevated glucose levels on EPCs (Chen et al., 2007), however it was also suggested that hyperglycemia may promote EPCs dysfunction indirectly through the induction of Reactive Oxygen Species (ROS) overproduction and increased oxidative stress (Callaghan et al., 2005). Moreover, anti-diabetic treatments were reported to improve the re-endothelialization capacity of EPCs from diabetic individuals (Gensch et al., 2007).

6.2 Insulin resistance (IR)

Approximately 80% of all T2DM coexist with IR (Zimmet et al., 2001). Several studies have proposed that IR may affect unfavourably the balance between endothelial injury and endogenous repair, promoting EDys and contributing to premature atherosclerosis (Dandona et al., 2003; 2004). Apparently, IR aids EDys perpetuation (Kim et al., 2006) by modulating vasculogenesis-associated EPCs capability of effectively promoting endothelium regeneration. Although the direct mechanisms by which IR alters EPCs functions are still unclear, it was recently suggested that the chronic inflammatory environment present in T2DM leads to insulin signaling defects in EPCs, thereby reducing their survival (Desouza et al., 2011). Additionally, it was also reported that after arterial injury in hemizygous knockout mice for the insulin receptor (IRKO), EPCs activities were altered and endothelial regeneration delayed. This defective endothelial repair could be normalized by transfusion of progenitor cells from insulin-sensitive animals, but not from insulin-resistant animals (Kahn et al., 2011). However, it is thought that EPCs biological modifications are mostly affected by IR in an indirect fashion, through the increase in ROS and also by the activation of pro-inflammatory cytokines (Cubbon et al., 2007; 2009; Houstis et al., 2006). In fact, IR states are closely linked to the increased production of ROS, which is a characteristic feature of IR and thought to play a causal role in its development (Houstis et al., 2006). The deleterious effects of oxidative stress in EPCs biological characteristics have been established and will be contemplated in section 6.4. Although further direct cause-effect between IR and EPCs alterations is currently under

investigation, it has been shown that treatments with insulin sensitizing drugs may improve EPCs functional parameters, independently of glycemic levels and/or redox status (Schoonjans & Auwerx, 2001). Nonetheless, further studies are necessary to clarify the molecular links between IR and vasculogenic impairment.

6.3 Hypertension

High blood pressure levels are associated with significant mechanical endothelial injury and dysfunction (Spieker et al., 2000). Disruption of endothelial homeostasis in hypertensive patients is thought to worsen their cardiovascular prognosis (Perticone et al., 2001) and contribute to increased blood pressure levels (Schiffrin, 2001). Alterations in vasculogenesis-related mechanisms have been associated to hypertension-induced EDys (You et al., 2008mi; Watson et al., 2008). A clinical study in patients with coronary artery disease (CAD) concluded that hypertension was a major independent risk factor predictor of impaired EPCs-induced migration (Vasa et al., 2001). It was reported that the functional activity of EPCs is reduced in experimental model settings and in hypertensive patients, due to increased EPC-induced senescence (Imanishi et al., 2005). Recently, it was shown that *in vivo* endothelial repair capacity of early EPCs was reduced in patients with pre-hypertension and hypertension, due to EPC senescence and impaired endothelial function, which potentially represents an early event in the development of hypertension (Giannotti et al., 2010). Although it is still unclear, hypertension does not seem to have a direct action on EPCs reduction of half-life, which may be caused by telomerase inactivation associated with the increase in oxidative stress associated with hypertension (Higashi et al., 2002; Imanishi et al., 2005; Touyz et al., 2004). Further, studies showed that in patients with arterial hypertension, no association was observed between the number of circulating vascular progenitor cells and hypertension, suggesting that cell mobilization may not be affected (Delva et al., 2007; Werner et al., 2005). Inconsistently, it was also reported that reduced levels of circulating CD34+VEGFR-2+ EPCs were detected in hypertensive patients as compared to normotensive individuals (Pirro et al., 2007). Lower levels of peripheral EPCs correlated with a downregulation in the homeobox A9 (HOXA9) gene expression, which is critical for endothelial commitment during progenitor cell maturation (Pirro et al., 2007). Further studies are required to clarify the role of hypertension in EPCs functions. Nonetheless, it was also reported that anti-hypertensive drugs, besides its blood pressure lowering effect, may also improve vascular function through EPC activation (Cacciatore et al., 2011; de Ciuceis et al., 2011; Yao et al., 2007).

6.4 Oxidative stress

Increased oxidative stress has been proposed as an important molecular mechanism for vascular complications associated with DM, IR, and hypertension (Aronson, 2008; Houstis et al., 2006; Yanai et al., 2008), by exerting a direct cytotoxic effect on the vascular monolayer (Griendling & FitzGerald, 2003). ROS may directly harm the vascular endothelium while superoxide reacts with NO to form peroxynitrite anion (ONOO⁻), a powerful oxidant (Griendling & FitzGerald, 2003; Kuzkaya et al., 2003). Diminished release of eNO caused either by excessive oxidative degradation or impaired local production has been implicated in endothelial lining damage and insufficient repair capability due to deficient EPCs mobilization/functional status (Craeger et al., 2003; Yao et al., 2006). In fact, oxidative stress-induced reduction of NO bioavailability represents the major mechanism leading to

impaired EPCs *in vivo* re-endothelialization capacity and *in vitro* function (Fleissner & Thum, 2010; Sorrentino et al., 2007). NO deficient release by the vasculature is thought to impair EPCs migratory function and colony-forming ability, indicating a central role for eNO activity in EPC biology in increased oxidative stress conditions (Hill et al., 2003). Additionally, it was recently shown in patients with metabolic syndrome (MetS) and CAD, that oxidative stress may directly induce DNA damage on EPCs, by promoting telomere shortening with a consequent increase in their senescence rate, which contributed to the progression of atherosclerosis (Fleissner & Thum, 2010; Satoh et al., 2008). Accordingly, anti-oxidant therapies, which exert cellular protective effects by directly scavenging ROS reducing their damaging action, can also improve EPCs functions and regenerative abilities (Marrotte et al., 2010).

7. EPCs and diabetic retinopathy (DR)

DR, the leading cause of visual impairment in the western world, will occur in the majority of T1DM patients and about 20–30% will advance to the blinding stage of the disease. It is expected that over 60% of patients with T2DM will develop retinopathy and with the global epidemic of obesity and subsequently of T2DM this predicament is likely to worsen (Fong et al., 2002). Retinal neovascularization in diabetes is stringently affected by alterations in the local microenvironment. Hyperglycemia damages retinal microvasculature, which results in increased permeability, blood and serum leakage to the extravascular space, and progressive decline in retinal blood flow; as well as closure of the retinal microvasculature leading to DR. Retinal ischemia and release of angiogenic factors stimulate the proliferation of microvessels, leading to proliferative DR (PDR). Dysfunctional new vessel growth destroys the normal retinal architecture and capillary leakage causes diabetic macular edema (DME), the principal cause of vision loss in diabetes (Li Calzi et al., 2010). Up until recently, angiogenesis was thought to be the only process governing aberrant diabetic retinal neovascularization. However, retinal ischemia-induced release of specific factors may stimulate both local growth of vessels and the mobilization of BM-derived EPCs, which contribute to the development of PDR. Initial studies in PDR experimental models have shown that EPCs could be recruited to retinal sites of ischemic injury, playing a role in the revascularization of the retina (Goon et al., 2007; Grant et al., 2002). Although the mechanisms underlying EPCs roles are still under evaluation, it was reported that they may be mobilized and recruited to the diabetic retina in response to local secretion of VEGF and SDF-1. Moreover, studies in PDR experimental models and in diabetic patients presenting this complication have shown that SDF-1 seems to be the most important chemokine involved in the mobilization of EPCs to the retina (Butler et al., 2005; Csaky et al., 2004). In addition, the concentration of SDF-1 increases with the severity of DR, as evaluated in vitreous samples of T2DM individuals (Butler et al., 2005). Recently, it was also proposed that retinal neuronal tissue could play a role in promoting EPCs-mediated neovascularization (Liu et al., 2010). This study has demonstrated that higher levels of the neurotrophins Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) are present in the PB of DR patients, but not in non-diabetic controls or DM-PAD patients. Additionally, a strong correlation between these neurotrophins and EPC levels in DR patients was found, suggesting that retinal ischemia serves as a signal to stimulate BM-EPCs through selected strong neurotrophic factors that are released into the systemic circulation. Recently, two reports conveyed more important information on the role of different

progenitor cells in various stages of DR in T1DM and T2DM patients (Brunner et al., 2009, 2011). In T1DM, it was demonstrated that in non-PDR patients there was a reduction in circulating EPCs, and that in PDR there was a dramatic increase of mature EPCs (Brunner et al., 2009). In addition, circulating EPCs of T1DM patients with PDR were reported to have increased clonogenic potential (Asnaghi et al., 2006). In T2DM patients with DR, circulating angiopoietic cells as EPCs, and mature EPCs had different regulations in PDR depending on each individual's macrovascular comorbidities (Brunner et al., 2011). Recent findings in diabetic patients have corroborated gathered experimental data, demonstrating that BM-derived CD133+ EPCs, as well as, CD14+ monocytes could be mobilized to diabetic epiretinal membranes, contributing to vasculogenesis in PDR (Abu El-Asrar et al., 2011). Taken together, these data strengthen the importance of EPCs in the development of human PDR, highlighting the crucial role played by both the local retinal and the systemic environment.

8. Modulating EPCs functions as therapeutic strategy

It seems paradoxical that diabetic vascular complications, as PDR and PAD, may both affect the same patient, and alterations in EPCs exhibit opposing roles. This contradictory puzzle will most certainly have an influence when considering the modulating of EPCs levels/functions as therapeutic intervention. It has been shown that several vasculoprotective agents provided with beneficial cardiovascular effects, such as statins, thiazolidinediones (peroxisome proliferators-activated receptor-gamma; PPAR- γ), and anti-oxidants have been shown to improve endothelium-dependent vascular function and prevent atherosclerotic disease progression, by restoring EPCs properties and actions (Chen et al., 2011; Kusuyama et al., 2006; Schoonjans & Auwerx, 2000). Additionally, and although there are no conclusive answers on the safety on EPCs therapies and of their potential undesired side effects, pre-clinical and clinical studies have highlighted that autologous transplantation of several stem and progenitor cell populations ameliorated diabetic peripheral vascular complications (Procházka et al., 2009; Zhou et al., 2007). Nonetheless, the dysfunction of endogenous EPCs may limit the feasibility and efficiency of this approach, since their biological features are altered, which reduces their capacity to significantly improve therapeutic neovascularization. In addition, one might not neglect that there are several EPCs subtypes, which raises the question on what might be the best reparative BM-derived EPCs population (Figure 1). Further, would EPCs-based therapies provide any beneficial effects in retinal neovascularization? As aforementioned, retinal new vessel growth associated to diabetes is dysfunctional, destroying the normal retinal architecture. So we may assume that improving diabetic EPCs functions/transplanting non-diabetic EPCs to the retina may promote the re-endothelialization of acellular capillaries and the elimination of retinal ischemia. In fact, if intra-retinal neovascularization could be harnessed at the appropriate stage, ischemia could be contained or reversed (Figure 2). However, since the intravitreal delivery of anti-angiogenic drugs may provide multiple benefits on DME and PDR (Arevalo et al., 2011; Chung et al., 2011), would the therapeutic use of EPCs be a better strategy for the treatment of DR? Many questions are still answered and in order to promote efficient EPCs-based therapies and to prevent harmful side effects, it is needed to go deeper into the molecular events accompanying alterations in diabetic vascular complications governing the diabetic-vasculogenic paradox.

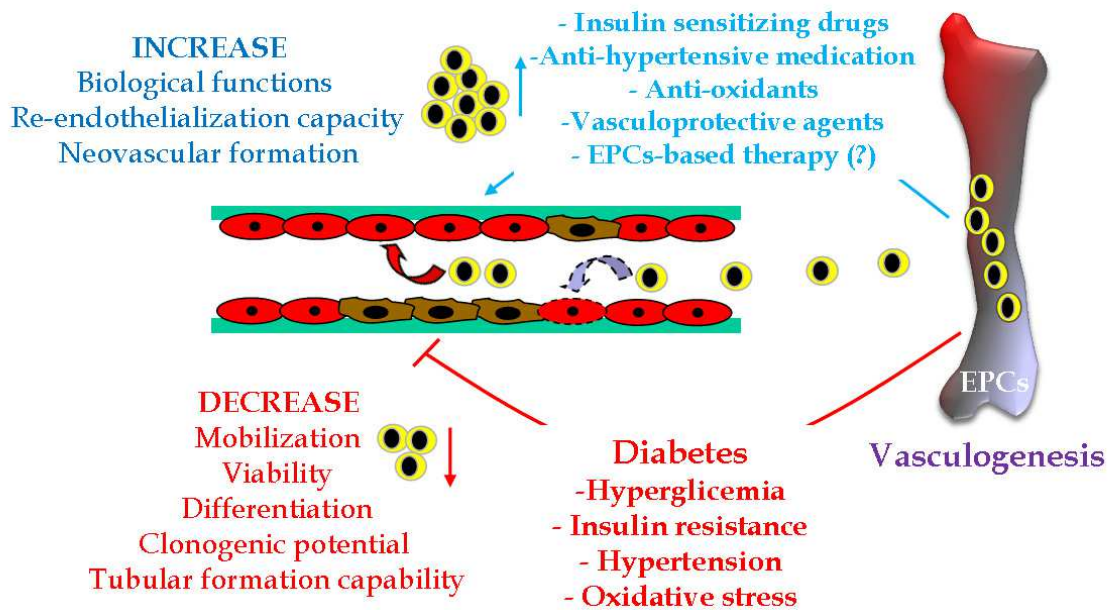


Fig. 1. Diabetes and peripheral vasculopathy. Diabetes related metabolic disorders decrease EPCs number and function being associated with impaired re-endothelialization and neovascular formation. Several agents may increase EPCs functions improving their re-vascularization capabilities. Adapted from Costa & Vendeira, 2007.

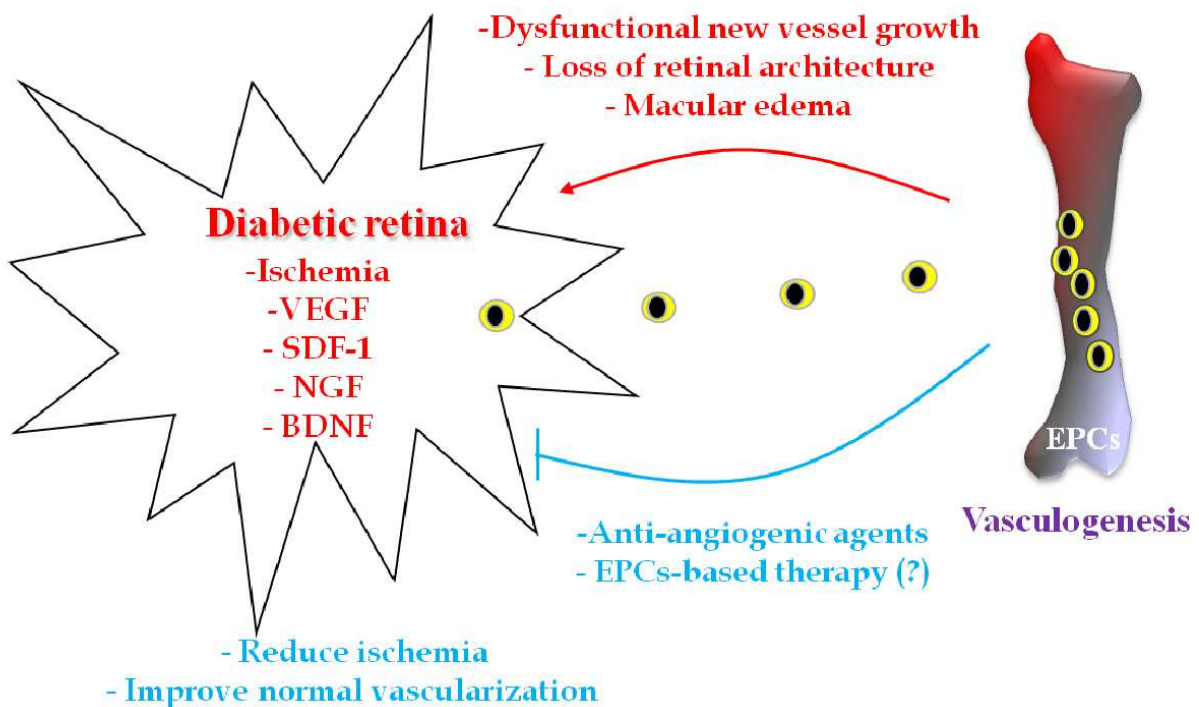


Fig. 2. Diabetic retinopathy. The ischemic retinal microenvironment and the production of vascular and neurotrophic factors promote increased EPCs recruitment and dysfunctional neovascular formation. Anti-angiogenic agents may improve retinal vascular architecture.

9. Concluding remarks

Even though EPCs constitute a relatively small percentage of circulating cells, they can specifically and effectively home to sites of injury and partake in the regeneration and repair of endothelial beds. Besides the initially identified population of CD34+AC133+VEGFR-2+ EPCs, other subsets of EPCs and progenitor cells with vascular repair capability have been described. We have discussed the most recent data demonstrating that dysfunctions of several EPCs subpopulations may have a prominent role in the pathogenesis of diabetes systemic and retinal vascular complications. Both the decrease and increase of neovascular formation in diabetes seem differentially regulated by dysfunctional EPCs, which respond selectively to the local depletion/accumulation of growth factors, explaining the reasons why peripheral ischemia cannot stimulate EPCs recruitment, in opposition to what occurs in the retina. Further studies are required to identify the beneficial effects/safety of EPCs-based therapies. Additionally, it is mandatory to investigate the efficient use of EPCs in promoting neovascularization in peripheral vascular disease and the abrogation of retinal ischemia and altered vascular architecture. This careful evaluation is crucial to further unveil the diabetic-vasculogenic paradox.

10. References

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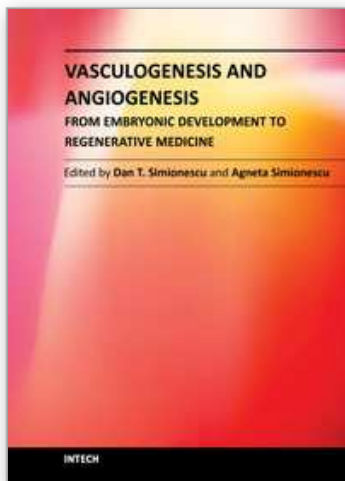
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Vasculogenesis is the process of new blood vessel formation during embryonic development of the cardiovascular system. This is followed by formation of a vascular tree and finally the cardiovascular system with the myriad of blood vessels that nourish all tissues and organs. Angiogenesis, on the other hand is the process by which new blood vessels take shape from existing blood vessels by "sprouting" of endothelial cells thus expanding the vascular tree. Both scenarios are based on activation, migration, proliferation and maturation of unique precursor cells. The study of blood vessel formation is an essential component of embryonic development, congenital malformations, degenerative diseases, inflammation and cancer and thus has widespread appeal to the biomedical field. Moreover, scientists are now harnessing this information for the purpose of building living blood vessel substitutes for replacement of diseased arteries and veins. This book highlights novel advances in the field of vasculogenesis and angiogenesis, including embryogenesis and development, regulation of progenitor cells, cancer and blood vessel regeneration. We consider this book a good initial source of information for graduate students, medical students and scientists interested in the intricacies of blood vessel formation, maturation, disease and replacement.

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