

Review

Endothelial progenitor cells in the natural history of atherosclerosis

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Abstract

Atherosclerotic diseases are responsible for a significant part of morbidity and mortality in western countries. According to the classical views, atherosclerotic lesions develop as the result of an inflammatory process initiated by endothelial damage. The discovery that bone marrow-derived cells participate in endothelial repair and new vessel growth has changed the pathogenetic models of cardiovascular disease. These cells, termed endothelial progenitor cells (EPCs), represent the endogenous endothelial regenerative capacity and the ability to form new collateral vessels. In this review we describe how quantitative and qualitative alterations of EPCs have a significant role in virtually all stages of the atherosclerotic process and in the clinical manifestations of the diseases: starting from the impact of risk factors on EPCs, through the mechanisms that link EPC reduction/dysfunction to plaque formation, and finally to the clinical syndromes. An attempt to diverge our attention from the vessel wall to the bloodstream reveals a central role of EPCs in atherogenesis.

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Contents

1. Introduction	46
2. The impact of risk factors on EPCs	47
3. The prognostic value of EPCs	49
4. EPCs in the progression of atherosclerosis	49
5. EPCs and the complications of atherosclerosis	50
6. Therapeutic implications: changing the natural history of atherosclerosis acting on EPCs	50
7. Warning and conclusion	51
References	51

1. Introduction

Atherosclerosis is a systemic inflammatory disease of the arterial wall [1]. Atherosclerotic plaque can critically reduce

blood supply to an organ, leading to symptoms such as angina pectoris, intermittent claudication, angina abdominis and renovascular hypertension. Furthermore, acute thrombotic or haemorrhagic complications of the plaque can result in arterial occlusion, leading to myocardial infarction or stroke. Therefore, atherosclerosis is a major issue in public health, as it associates with significant morbidity and mortality. A damage to the inner layer of the arterial wall, the endothelium, constitutes the *primum movens* of the process that leads to plaque formation. Classical risk factors for atherosclerosis

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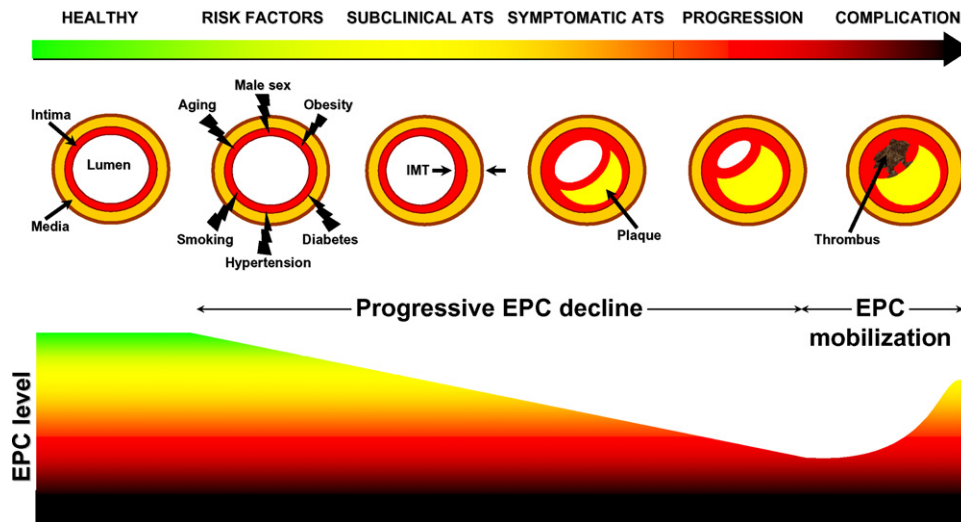


Fig. 1. Regulation of EPCs following the natural history of atherosclerosis. ATS, atherosclerosis; EPC, endothelial progenitor cells; IMT, intima-media thickness.

concur to determine endothelial damage, but mechanisms are still incompletely understood.

Noteworthy, the endothelium has the ability to repair itself [2]. When a small area of the intima is removed experimentally, endothelial cells at the edges of the lesion proliferate and migrate toward the centre, because of lost contact inhibition. If the endothelium is young and healthy, the local repair process is complete and the intimal layer is reconstituted. On the contrary, if the endothelium is older or it receives the assaults of one more risk factors, such as cholesterol, hypertension, or hyperglycemia, local repair is defective and a plaque may develop as the result of an inflammatory process elicited mainly by macrophage accumulation [3]. In the last 10 years, it has become apparent that endothelial repair is driven not only by local cells, but also with the contribution of circulating cells [4]. Cells with the ability to repair the endothelium have been termed endothelial progenitor cells (EPCs). EPCs derive from the bone marrow and can be mobilized to the peripheral circulation upon a variety of stimuli including tissue ischemia through the release of growth factors [5]. Once in peripheral blood, EPCs constitute a pool of cells that can actively repair the endothelial layer by forming a patch at sites of intimal damage [6,7]. These processes are mediated by interactions between EPCs and mature endothelial cells through the expression of adhesion molecules and the release of chemokines [8–10]. The actual quantitative contribution of EPCs to the endothelial homeostasis is not known precisely, but studies that used chimeric animals carrying green fluorescent protein (GFP)-positive bone marrow show that EPCs are critical for endothelial repair and that EPC depletion impede complete regeneration [11–13]. Additionally, EPCs are also integrated into the endothelium of the nascent vasculature, sprouting from existing vessels (neovascularization) or developing *de novo* mostly as the result of an inflammatory-like process (neovascularization) [14]. Compensatory angiogenesis is a clue event triggered by the critical reduction in blood

flow, and determines the extent of residual tissue ischemia. The ischemic organ, through the release of growth factors and cytokines, stimulates bone marrow to release EPCs which, in turn, specifically home at the damaged sites through the expression of chemokine receptors, and finally stimulate new vessel growth [5]. Therefore, EPCs represent the fulcrum of a negative compensatory feedback loop which maintains vascular homeostasis. Thanks to their comprehensive role in endothelial regeneration and compensatory angiogenesis, EPCs are currently considered an integrated component of the cardiovascular system that is subject of intense research and debate [15]. Not only the extent of the EPC pool is an indicator of vascular health, but also normal functions of EPC are required for adequate homeostasis. Functional EPC properties are explored *in vitro* using standardized assays for proliferation and colony formation, migration in a Boyden-like chamber, adhesion to a mature endothelial monolayer, and incorporation into vascular networks on matrigel. Number and function of EPCs are indeed two sides of the same coin and their alterations have been related to cardiovascular disease and atherosclerosis [16]. Moreover, quantitative and qualitative EPC properties are co-regulated by the same molecular pathways, so that EPC decrease is usually associated with dysfunction, and EPC increase is usually associated with enhanced function. Remarkably, there are exceptions to this rule, which merit attention, because could potentially disclose relevant mechanisms of EPC regulation.

2. The impact of risk factors on EPCs

The natural history of atherosclerosis begins early, when predisposing factors appears. Almost all classical risk factors for atherosclerosis have been shown to exert detrimental effects on EPC number and function. Currently, negative

EPC modulation is considered one mechanism by which risk factors worsen cardiovascular health [17].

Age is a predominant determinant of the extent of the circulating EPC pool: the EPC level is strongly negatively correlated with age and, as subjects get older, function of their EPC progressively decline, in terms of survival, differentiation, proliferation and migration [18–21]. This phenomenon is likely part of the global aging process, which limits the functional reserve of virtually all organs and tissues, including bone marrow. Nonetheless, as age increases, other risk factors, such as hypertension and diabetes, become more prevalent and may independently impact on EPC biology.

In animal models of hypertension, as well as in subjects with essential hypertension, EPCs become precociously senescent and dysfunctional [22]. Higher blood pressure levels are associated with lower EPC levels in the general population [23], in diabetic subjects [24] and in coronary patients [25]. Hyperactivity of the rennin–angiotensin–aldosterone system (RAAS) as been recognized as one link between hypertension and altered EPC biology. Both *in vivo* and *in vitro*, angiotensin II (At-II) supplementation negatively modulated EPCs through induction of oxidative stress, an effect that was prevented by angiotensin receptor blockers (ARBs) [22,26]. Further, *in vitro* aldosterone inhibited EPC generation and differentiation by attenuating release of vascular endothelial growth factor (VEGF) and Akt signalling, while the effects were prevented by spironolactone and antioxidants [27]. With this background, it is tempting to speculate that endothelial dysfunction, arterial stiffness and vascular rarefaction seen in patients with hypertension may be attributed, at least in part, to exhaustion of the EPC pool.

Extensive human and animal studies have shown that type 1 and type 2 diabetes mellitus are characterized by profound EPC reduction and dysfunction [28,29]. Hyperglycaemia itself together with the resulting oxidative stress probably account for this alteration [30]: through a defective PI-3K/Akt pathway, high glucose dampens EPC differentiation and ability to integrate into vascular structures and induces apoptosis. The imbalance in FoxO phosphorylation/acetylation and its nuclear translocation likely mediated the downstream effects of high glucose by modulating proapoptotic gene expression. Remarkably, those alterations were prevented by treatment with benfotiamine, a thiamine analogue that smoothes upstream mediators of glucose toxicity, such as the hexosamine pathway, non-enzymatic glycation, and protein kinase C activation [31]. As a clinical counterpart, reduction of EPC is more pronounced in subjects with higher glucose levels [32] and accumulation of advanced glycation endproducts (AGEs) impairs EPC function and may alter the microenvironment of bone marrow and target tissues [33]. In support of this notion, diabetic subjects with a longer disease duration and a higher level of glycohaemoglobin had lower levels of EPCs in their peripheral blood. Diabetic subjects have an impressive risk of developing accelerated atherosclerosis and myocardial or peripheral ischemia. This has been recently attributed to impaired endothelial regeneration and

defective compensatory angiogenesis [34,35], both of which may be related to EPC decrease and dysfunction.

Cigarette smoking is a potent inducer of vascular dysfunction and atherosclerosis. Smoking causes exhaustion of circulating EPCs and smoking cessation rapidly favours restoration of a normal EPC pool [36]. Additionally, EPCs isolated from healthy smokers exhibit a global functional impairment (proliferation, differentiation, adhesion, migration and tubulization) compared to non-smokers [25,37]. Smoking habit is also a cause of chronic obstructive pulmonary disease (COPD). COPD has been associated with EPC depletion [38], especially when secondary cardiovascular abnormalities are present, which was more pronounced in smokers than in non-smokers [39]. EPCs have been disclosed to be sensitive to oxidative stress, and cigarette smoking in fact contains a deadly cocktail of toxic compounds that act as mutagens and oxidants: those substances overwhelm the effects of nicotine, which has a surprising potential to mobilize EPCs and enhance their function [40].

Cholesterol is the major component of the atherosclerotic plaque and hypercholesterolemia remains so far the strongest risk factor for atherosclerosis. Consistently, higher cholesterol level was associated with EPC depletion independently upon other risk factors. Moreover, proliferation, migration and *in vitro* vasculogenesis by EPCs were impaired in hypercholesterolemic subjects [41]. In another study, EPC culture yield from healthy subjects was strictly related to the lipid profile, especially HDL [42]. Indeed, supplementation of HDL prevented EPC apoptosis, increased eNOS expression in culture [43], and promoted progenitor cell-mediated endothelial repair *in vivo* [44]. By converse, treatment with atherogenic lipoproteins (LDL and VLDL) reduced the number of EPC colony units, while oxidized LDL reduced EPC survival, differentiation and vascular network formation [45–47].

Sedentary lifestyle negatively impacts on cardiovascular health in terms of body composition, cardiac function, lipid profile and carbohydrate metabolism. By converse, physical exercise counteracts those alterations and help maintaining vascular homeostasis. Interestingly, physical training induced mobilization of EPCs from bone marrow to peripheral blood in normal mice [48], healthy subjects [49] and in subjects with coronary artery disease [50,51]. This phenomenon may be transient and the specific type of exercise required to mobilize EPC (aerobic or anaerobic) is still not clear [52]. Even if we currently do not know whether sedentary subjects have indeed a lower steady-state level of EPC than training subjects, at present exercise activity remains the only known lifestyle intervention to stimulate EPCs [53].

Linked to sedentary lifestyle and excessive caloric intake, obesity is characterized by EPC defect in relation to waist circumference. Adipokines derived from visceral fat, such as leptin [54] and TNF-alpha [55], play negative effects on cultured EPCs and may be one link between obesity and EPC depletion. Remarkably, visceral obesity is typically associated with other risk factors, clustered in the metabolic

syndrome (MetSyn). The diagnosis of MetSyn pinpoints a cardiovascular risk that is higher than the sum of its parts. Parallely, clustering components of the MetSyn reduce EPC level in a synergistic way, that is more than if the negative impact of each component was additive [56]. This is because the underlying pathophysiological link, insulin resistance, is probably itself a determinant of the EPC pool, as it is and independent risk factor for cardiovascular disease.

Male gender is the archetypal factor associated with increased risk of atherosclerotic disease: males develop atherosclerotic lesions on average 10 years before females. Again, EPCs may have a role in this gender-related dichotomy, as the female hormones estrogens have been recognized as important regulators of EPC number and function [57].

Among classical risk factors, family history of cardiovascular disease has not been clearly related to EPC alterations. However, the EPC level distributions of subjects with and without with risk factors for cardiovascular disease have overlapping tails, suggesting that there should be other determinants. Genetic background is likely to be involved in the determination of both the steady-state EPC pool and the response to injury. For instance, polymorphism of the stromal-derived growth factor (SDF)-1 influences the ability to mobilize EPCs from bone marrow to peripheral blood [58]. Observational studies enrolling subjects with a positive family history of precocious myocardial infarction in first-degree relatives will provide excellent pathophysiological insights into genetic and environmental EPC regulation.

Finally, besides classical risk factors, also emerging risk factors, such as low-grade inflammation [59,60] and hyperhomocysteinemia [61] have been related to alterations in EPC number and/or function. Therefore, part of the residual variation of EPC that is not explained by classical risk factors, age and sex, may be attributed to occult biological phenomena that silently increase cardiovascular risk. This is why EPC count itself is going to become a novel surrogate marker of risk [62,63].

3. The prognostic value of EPCs

EPC levels decrease in parallel with the presence of risk factors. The extent of the EPC pool negatively correlates with cumulative indexes of cardiovascular event risk, such as the Framingham risk score, and multiple risk factors act synergically in reducing EPC, as in increasing risk [25,56]. Two important studies have confirmed the independent prognostic value of EPCs. Werner et al. have demonstrated that cardiovascular event rate at 1 year increases in parallel with decrease in baseline EPC level in patients with angiographically documented coronary artery disease, after adjustment for known confounders [64]. Almost simultaneously, Schmidt-Lucke et al. showed that reduced level of EPCs independently predicted atherosclerotic disease progression in a mixed population of healthy subjects and coronary patients [65].

These studies support clinically the relevance of the endogenous EPC pool as an indicator of the vascular regenerative capacity.

4. EPCs in the progression of atherosclerosis

Not only EPC count reflects cardiovascular risk and predicts future events, but is also directly related to disease severity.

Subjects with subclinical atherosclerosis, defined as an increased carotid intima-media thickness (IMT), have a lower pool of EPCs than subjects without signs of atherosclerosis, independently upon risk factors and C-reactive protein [23]. IMT is considered a strong biomarker of cardiovascular risk: it correlates closely with the anatomical vascular remodelling, and its measurement is accepted as the best way to detect early atherosclerosis in asymptomatic individuals. Therefore, besides the effects of risk factors, EPCs further reduce as initial atherosclerosis develops. As atherosclerosis is a systemic disease and it is expected to affect at the same extent all major vascular beds, symptoms will develop earlier in those tissues supplied by small arteries. Indeed, the most precocious form of clinically evident atherosclerosis, that is erectile dysfunction, is itself associated with a more profound EPC reduction than would be attributable to classical risk factors [66]. The resulting pathogenetic concept is that EPC depletion, conveyed by clustering risk factors, reduces the ability to repair the endothelium, thus triggering subsequent steps in the development of the atherosclerotic plaque.

The logical implication of this model is that lower is the EPC level, more diffuse and severe should be the anatomical atherosclerotic burden. In fact, studies demonstrating a linear correlation between EPC count and disease severity have become available. In asymptomatic subjects, EPC decrease was related to atherosclerosis severity assessed at the carotid, aortic and femoral sites [67]. In patients with type 2 diabetes, EPC level was negatively correlated with both the carotid atherosclerotic burden and the clinical stage of lower limb atherosclerosis obliterans [68]. In another study, circulating EPC count was an independent determinant of the severity of angiographically-detected coronary artery disease [69]. Noteworthy, there have been one report showing that ischemic heart disease is associated with dysfunctional but not reduced bone marrow-derived EPCs [70], thus suggesting that different cell sources may yield different results. Taken together, these data support the notion that the level of circulating EPCs is a direct indicator of the atherosclerotic burden in virtually all arterial districts.

When the atherosclerotic plaque progressively grows until it critically reduces blood supply to the target tissue, chronic ischemia develops: this is the case for stable angina and leg claudication. Thereafter, arterial collateralization becomes the only way to overwhelm vascular obstruction and, again, the contribution of bone marrow-derived EPCs is critical.

Interestingly, it has been shown that EPC level is correlated with the coronary collateral flow index, a measure of the collateral support in the coronary circulation [71]. Hence, it is easy to anticipate that subjects with a depleted EPC pool have an impaired ability to form collaterals and fail to compensate for the presence of a critical stenosis. Maybe this is the explanation for the higher incidence of major cardiovascular events in subjects with lower EPCs despite adjustment for coronary disease severity [64]. Similarly, it explains why patients with ischemic foot lesions display further EPC decline than patients without lesions despite comparable atherosclerotic involvement [68]. Consequently, depletion of circulating EPCs contributes to both endothelial dysfunction, as an early event in the atherogenetic process, and to poor collateralization, as a late event leading to the clinical manifestations of atherosclerosis and cardiovascular disease progression. From this picture, EPC count reveals as the prototype of a novel class of cardiovascular biomarkers: not only EPCs are crucial in maintaining endothelial integrity and vascular homeostasis, but their amount in peripheral blood is a strong surrogate measure of risk and a mirror of regenerative capacity and atherosclerotic burden [62]. A similar comprehensive role had not been depicted before, not even for other well-established markers, such as C-reactive protein [72], and strengthens the notion that EPC alterations are pathogenetically linked to cardiovascular disease.

5. EPCs and the complications of atherosclerosis

The last event in the natural history of the atherosclerotic disease is plaque complication: plaque haemorrhage, rupture and thrombosis lead to vascular occlusion, which means myocardial infarction or stroke. Despite all the history thus far has been characterized by progressive EPC depression, an extreme attempt to increase EPCs is now carried out to cope with the acute event (Fig. 1). Animal studies have definitively demonstrated that tissue ischemia upregulates many growth factors and cytokines, such as VEGF and SDF-1, which reach the bone marrow and stimulate the release of EPCs through eNOS and MMP-dependent pathways [5,73–75]. Then, their specific homing at the damaged site is directed by the interaction between chemokines produced locally and specific receptors on EPCs [76]. Enhancement of EPC mobilization limits the resulting damage, while inhibition of mobilization potentiates it [73,77]. In humans, myocardial infarction, unstable angina and direct vascular injury are followed by a sudden increase in circulating EPCs, while their level returns to basal after 1–2 weeks [78–82]. This reaction should limit tissue damage and promote early regeneration via a homeostatic negative feedback loop. In fact, it has been shown in patients, that the extent of EPC mobilization after acute myocardial infarction is an independent predictor of improvement in ventricular function at 1-year follow-up [83]. However, it is not known whether all patients with multiple risk factors and advanced atherosclerosis, which have a severe

depletion of their EPC pool, can still mobilize EPC after an acute event [17]. Interestingly, NO bioavailability, which is typically reduced in these patients, has revealed critical in the process of ischemic mobilization of EPCs from bone marrow [73]. Again, EPC regulation during myocardial infarction not only reflects the endogenous regenerative response, but more generally mirrors vascular health. Accordingly, diabetic rats completely lose the ability to upregulate EPCs after hindlimb ischemia, an effect that is restored by correction of hyperglycaemia [84]. This notion also provides a mechanistic insight into the myocardial protection conveyed by a tight glycometabolic control during acute coronary syndromes [85]. However, the functional integrity of mobilized EPCs is still matter of debate. While it was initially demonstrated that mobilizing stimuli increase functional EPCs in patients with multiple risk factors [86], recently, it has been suggested that events accompanying EPC mobilization, such as cleavage of CXCR4, may impair transiently their migratory capacity [87]. This represents the most relevant example in which number and function of EPCs are differentially modulated; it also strengthens the key role of the SDF-1/CXCR4 axis for EPC function.

6. Therapeutic implications: changing the natural history of atherosclerosis acting on EPCs

Importantly, there are various ways to increase circulating EPCs and improve their function. First, treatment of modifiable risk factors is able to restore the EPC pool. Smoking cessation [36], reducing blood pressure with angiotensin-converting enzyme (ACE) inhibitors [24], lowering blood glucose levels with insulin [58] and treating hypercholesterolemia with statins [73], all can induce a relevant increase in the levels of peripheral blood EPCs. It is still not known whether weight loss itself has positive effects on EPCs in obese subjects: studies on this topic will be welcome. Certainly, age is a potent risk factor that we cannot modify. However, the increased risk associated with aging in the female population is mainly attributed to the deficiency in sexual hormones after menopause. The discovery that estradiol deprivation downregulates EPCs and that estrogen replacement restores the EPC pool [13], together with the observation that estradiol enhances recovery after myocardial infarction and reendothelization after injury through EPC recruitment [88], is a novel rationale for hormonal therapies in menopausal women. Besides estrogens, many other pharmacological compounds display positive EPC-modulating properties [89]. Statins themselves potently stimulate EPC mobilization and differentiation and enhance EPC functions. Remarkably, those effects are independent upon lipids, suggesting that EPCs represent probably an outstanding target of the so-called “statin pleiotropy” [90]. Even if in steady-state conditions, statin therapy is associated with reduced EPCs in coronary patients [91], small clinical trials suggest that statins can increase EPCs also in patients with multiple risk factors

and established disease [92]. Thiazolidinediones, a class of PPAR- γ agonists used in the treatment of insulin-resistant type 2 diabetics, have been shown to favourably modulate EPCs *in vivo* and *in vitro* [93–95]. Importantly, those effects were independent upon amelioration of insulin sensitivity and decrease in blood glucose levels, suggesting a direct effect of glitazones on EPCs. Thus, besides correction of risk factors, we have at present different ways to increase EPCs using known and handy drugs that are already largely prescribed to patients with cardiovascular risk factors or established disease. Curiously, those drugs are known to be provided with pleiotropic effects that confer cardiovascular protection and slow the progression of atherosclerosis. A step further should be risk stratification on the basis of EPC levels together with classical risk prediction, upon which to decide how much intensively an individual patient should be treated.

More sophisticatedly, there are pharmacological approaches not routinely used in cardiovascular patients, which have the potentiality to stimulate endogenous EPCs. Growth factors that can naturally mobilize bone marrow progenitor cells to peripheral blood have been tested for their ability to treat various atherosclerotic diseases. While favourable results have been reported in peripheral arterial disease, such as improvement in limb perfusion [96], limited benefits were shown in patients with myocardial infarction [97,98], although men seemed more susceptible to favourable effects [99]. Moreover, doubts remain regarding the functional integrity of EPCs mobilized by those pharmacological agents [87].

Finally, many clinical trials of cell therapy have shown that transplantation of autologous EPCs or other cellular pools enriched with vascular progenitors is feasible in both coronary and peripheral atherosclerotic diseases. The forerunner TOPCARE-AMI trial showed improvement in left ventricular ejection fraction (LVEF) and decreased end-systolic volume 1 year after transplantation of autologous bone marrow cells or EPCs in patients with acute myocardial infarction [100]. Despite some inconsistencies, more recent controlled trials have shown marginal but significant improvement in LVEF in patients receiving bone marrow cells versus control patients already receiving state-of-the-art therapies, with the most favourable risk-benefit profile in patients with worse baseline LVEF [101]. Also in the setting of chronic ischemic heart disease, patients transplanted with bone marrow cells had a greater increase in LVEF compared with the control group [102,103]. In the setting of severe peripheral atherosclerosis with critical limb ischemia, two major trials have shown the even more enthusiastic effects of cell therapies. In the TACT study, implantation of mononuclear bone marrow cells led to improvement in surrogate indexes, such as ankle-brachial index (ABI) and transcutaneous oxygen pressure, as well as in hard endpoints, such as pain, ulcer size and amputation [104]. Interestingly, Huang et al. have reported almost identical results in diabetic patients with atherosclerosis obliterans using peripheral blood progenitor cells mobilized with granulocyte colony-stimulating factor

[105], which may represent a more easily accessible source of progenitor cells. Even if some doubts remain regarding the best source of cells and the optimal patient selection, stringent experimental data and novel clinical trials demonstrate that autologous EPC therapy really have the potential to modify the natural course of atherosclerosis.

7. Warning and conclusion

The methods employed in the study of EPCs are complex and not uniform [106,107]. Quantification of circulating EPC is generally performed using flow cytometry [108]: the gold standard EPC phenotype relies on the expression of the immature antigens CD34 and CD133 and of the endothelial-lineage marker KDR (VEGFR-2). Even if an excess of different antigenic definitions have been used and disagree exists regarding the optimal antigenic characterization, CD34⁺KDR⁺ is still the most credited phenotype. Moreover, EPCs can be counted also after culture isolation, and there is no definite demonstration that the direct *ex vivo* cytometry and the *in vitro* culture system yield consistent measures of the actual EPC pool. While methods to explore EPC function are generally standardized, EPCs isolation itself is not uniform [109]: in our opinion, regardless of the precise phenotype of origin, all putative isolated EPCs should fulfil some key criteria, such as self-renewal capacity, eNOS expression, and the *in vivo* ability to incorporate into neovessels [110]. Unfortunately, an albeit partial lack of consensus sometimes makes disparate studies poorly comparable and, in some cases, inconsistent. Therefore, in this review we have intentionally abstained from methodological disquisitions to emphasize the comprehensive roles of EPC in atherosclerotic diseases, reporting studies which appear as much comparable as possible. The resulting picture is amazingly harmonic and places EPCs at the centre of the atherosclerotic process: like a Copernican revolution, no more blood cells are passive bystanders, rather are the new stars.

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