Endothelial Progenitor Cells as a Therapeutic Option in Peripheral Arterial Disease

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Abstract  Background: Endothelial progenitor cells (EPC) are a subpopulation of bone-marrow mononuclear cells that are capable of generating new blood vessels in areas of ischaemia or infarction. This review examines the regenerative potential of EPC to ameliorate peripheral ischaemia.

Methods: An online search was done using OVID Medline Search, PubMed, and Cochrane Review Database, for all reviews and original articles in English concerning progenitor or bone-marrow mononuclear cells.

Results and conclusion: There are many controversies in EPC research, especially in the areas of identification, characterization, and therapeutic use. Both animal and human studies have shown benefits from using EPC to combat peripheral arterial and cerebrovascular disease. To bring EPC into wider clinical use, larger controlled clinical trials and better methods of augmenting EPC function and lifespan are required. Until then EPC should be used under robust trial conditions with ethical approval.

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table

Introduction

Successes with stem cell therapies in the treatment of haematological disorders over the past four decades, coupled with developments in our understanding of the physiology of vascular remodelling, have fuelled much interest in regenerative medicine and therapeutic restoration of the damaged organ. The classical theory of vasculogenesis postulates that the adult vascular tree is formed during foetal development, and vascular repair in the adult occurs by mitotic division and migration of mature endothelial cells from pre-existing blood vessels. Developments over the last decade or so, however, suggest that postnatal vasculogenesis (called neangiogenesis) is brought about by circulating progenitor cells, capable of differentiating into mature blood vessel endothelial cells.1

One of the breakthroughs came in 1997 when Asahara et al. isolated cells in the peripheral blood that were capable of differentiating into mature endothelial cells in vitro.1 They also showed in vivo mobilization and incorporation of these autologous cells into sites of tissue

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ischaemia and neovascularization in a rabbit hindlimb ischaemia model. This was the first step in identification of the endothelial progenitor cells (EPC) and confirmed that adult neoangiogenesis was different from embryonic vasculogenesis in that it did not rely exclusively on the division of native endothelial cells in the blood vessels.

The term progenitor refers to a population of stem cells that remain in a state of growth arrest in the body while retaining the capacity to enter cell cycle on demand and differentiate into progeny. Progenitor cells are different from stem cells in their inability to renew themselves indefinitely. Therefore they are believed to become obsolete after a limited number of cell divisions and seem to lose their proliferative potential with maturity, with the highest proliferative potential found in cells derived from umbilical cord blood compared to peripheral blood in humans. Bone marrow is believed to be the major site of origin of EPC — bone-marrow-derived cells (BMDC) — but recent reports suggest that EPC can be derived from other sources such as liver, epicardium and intestine, and non-BMDC are probably more important in the setting of postnatal vasculogenesis.

EPC in general facilitate neovascularization by translocating to sites of vascular ischaemia, differentiating into endothelial cells, and increasing collateralization. They have been shown in ex vivo cultures to form three dimensional tubular structures (neoangiogenesis) that could rescue tissue from ischaemia, and preserve function by physical limitation of the infarct zone.

The majority of translational work investigating the therapeutic potential of EPC has been related to myocardial diseases — coronary artery disease (CAD), chronic left ventricular dysfunction and ischaemic cardiomyopathy — with some evidence also emerging from peripheral arterial disease (PAD). Furthermore, the adjuvant potential of the EPC as a cardiovascular risk biomarker has been suggested, based on the inverse correlation between the number and migratory activity of EPC with risk factors for CAD, including diabetes mellitus (DM), hypercholesterolaemia, cigarette smoking, age and hypertension. This review examines the relevant physiology of the EPC for a clinician and provides an insight into the evidence behind the potential of EPC therapy to combat peripheral ischaemia.

**Methods**

An online search was done using OVID Medline Search, PubMed, and Cochrane Review Database from January 1997 to August 2008, for all articles in English using keywords endothelial progenitor cells, circulating or bone-marrow-derived progenitor cells, neoangiogenesis. The clinical trials were searched separately for each disease process by meshing keywords peripheral arterial disease, coronary artery disease and stroke with endothelial progenitor cells. All the abstracts were reviewed by at least 2 reviewers and 409 articles shortlisted for detailed study, of which 49 were included in this paper. Articles included were those detailing clinical trials and translational research investigating the potential of bone marrow or circulating EPC for neovascularization. For clinical studies, only papers relating to human trials in atherosclerosis were selected. This was the only criterion used for exclusion of clinical trials because a meta-analysis is not attempted, given the diversity of protocols and outcomes present. In case of disagreement between the first three authors, the senior author (SHV) made the final decision. SHV independently reviewed the collected data for consistency and relevance.

**Problems with Characterization and Quantification of EPC — Impact on Clinical Application**

At present there is no single best approach to characterize or quantify EPC. This problem is well recognized and arises from a combination of issues. There are various sub-cATEGORIES of stem cells such as haematopoietic stem cells (HSC), EPC, epithelial cell lines, cancer stem cells, neural stem cells and very small embryonic-like stem cells (VSEL). These cell lines share a variety of cell-surface markers and the markers tend to change in accordance with the developmental hierarchy of stem cells: embryonic stem cells have different surface markers as compared to differentiated cells higher up the chain.

The source of an EPC influences the identifying markers. Cardiac muscle, adipose tissue, liver and intestinal tissues, as potential sources of extractable cells with EPC characteristics, often have their own identifying markers. These labels are useful for identifying cells from specific anatomical ‘niches’ in the peripheral blood but may contribute to inaccuracies in the estimation of the cumulative effect of stem cell therapies; the calculation of the number of circulating EPC in healthy versus diseased individuals may also be misleading.

While no single cell-surface marker has been found to be absolutely specific for EPC, there are several markers which clearly identify functional EPC populations. These include CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) and vascular endothelial cadherin. These markers are found on mature endothelial cells as well and so may lead to inclusion of a more mixed cell population including mature cells with a lower proliferative capacity. They have also been found to include cell populations that participate in angiogenesis but do not differentiate into endothelial cells in vitro. These cells, expressing blood monocyte markers CD14 and CD45, are thought to facilitate neoangiogenesis through secretion of angiogenic factors (paracrine effect). The inclusion of mature and non-EPC phenotypes may mask the potency of progenitor cell therapy. For this reason, markers with a higher specificity for immature cell-types such as CD133 may be helpful. At present the best approach to quantify and characterize EPC appears to be to include as many markers as possible.

As previously stated, EPC tend to lose their proliferative potential as they mature, with the highest proliferative potential being found in cells from umbilical cord blood. Different cell-surface markers are associated with different stages of maturation of EPC in their natural cell cycle: immature EPC express cell-surface markers that closely resemble HSC such as CD133. These markers are gradually succeeded by a more endothelial phenotype, expressing CD31, vascular endothelial cadherin and von Willebrand factor. This problem is further compounded by the ability of cells of one lineage to differentiate into other cell ...
lineages — transdifferentiation. It is primarily the peripheral blood mononuclear cells (PBMCN) that have been shown to differentiate into endothelial as well as cardiac and neural phenotypes in different culture media in vitro. No studies have yet demonstrated progenitor cells with a specific endothelial phenotype to be capable of differentiating into alternate lineages. The similarities in phenotype and overlap in function of PBMCN may be explained by a common stem cell population for EPC, HSC, and other progenitor cells. EPC can thus be considered a differentiated form of this primary progenitor cell with the greatest propensity for further maturation into endothelial cells. It is as yet unclear to what extent each progenitor cell is committed to its mature counterpart, but it is likely that potential for transdifferentiation into alternate lineages exists in immature EPC or embryonic stem cells, which are lower in maturity hierarchy.

The other important aspect of EPC therapy is to characterize the angiogenic capacity of EPC. The three most commonly used techniques for this purpose include: the murine hindlimb ischaemia for in vivo animal studies, maigel tube formation assays for estimating ex vivo angiogenic potential by measuring the EPC capacity to form three-dimensional structures (tube formation), and ex vivo migration assays for estimation of migratory capacity. Although the ability of EPC to stimulate neoangiogenesis seems apparent, different pre-clinical studies base their results on different aspects of EPC function, making collative analysis of the data difficult. Similarly there have always been suspicions about the actual impact of incorporation of EPC-derived endothelial cells into neovessels, and it now seems more likely that the paracrine effects of EPC therapy play a major role in neoangiogenesis. EPC numbers and function are augmented by a variety of growth factors such as VEGF, stromal-derived factor-1, insulin-like growth factor-1, hepatocyte growth factor, and thymosin β4, which also encourage adjacent endothelial cells to stimulate angiogenesis. Another confounding factor is the use of gene therapy to enhance functional potency of EPC and growth factor therapy by reducing the requirement of bone marrow for ex vivo expansion of EPC prior to inoculation or transplantation. These therapies have their own paracrine effect and this may again lead to inaccuracies in estimating the actual impact of EPC on the observed functional outcome.

**Relationship of EPC to Cardiovascular Risk Factors**

Cardiovascular risk factors impair the function of EPC by age-related exhaustion of the bone-marrow niche and by EPC senescence. EPC senescence is a term used to describe progenitor cells which have lost their ability to proliferate, migrate and differentiate into mature cells. Therefore, senescence accounts for reduced EPC function and numbers. The number and migratory activity of EPC have been reported to be reduced in most of the major cardiovascular risk factors such as DM, hypertension, hypercholesterolaemia, increasing age, and cigarette smoking. Most likely, the observed impairment of EPC in patients with cumulative cardiovascular risk factors is attributable to their reduced regenerative capacity. These results have been confirmed in the 'Endothelial Progenitor Cells in Coronary Artery Disease' (EPCAD) study, which demonstrated that the level of circulating CD34+/KDR+ EPC predicts the occurrence of cardiovascular events and cardiovascular death. In patients with arterial hypertension, systolic blood pressure inversely correlates with the number of circulating CD133+ and CD34+/KDR+ EPC but the number of colony-forming units is not impaired by arterial hypertension. Impaired EPC activity in hypertensive patients is proposed to occur via angiotensin II-mediated acceleration of EPC senescence and angiotensin II type 1 receptor antagonists such as valsartan increasing the number of regenerating EPC.

In vitro studies have demonstrated that culture media conditioned by EPC from patients with Type 1 DM exhibit reduced angiogenic capacity and a reduced capacity to participate in tubule formation. The number of EPC has also been shown to correlate inversely with Hb A1c levels, providing an insight into the relation between tight glucose control and EPC function. Similarly, it has been demonstrated that blood cholesterol level is inversely correlated to EPC number and function. Oxidized low-density lipoproteins (LDLs) have been shown to impair the proliferative, migratory, adhesive and in vitro vasculogenic capacity of EPC with a dose-dependent effect. One of the explanations for this effect is the inhibition of VEGF-induced EPC differentiation through the dephosphorylation of Akt (a protein kinase) by oxidized LDLs. This is important clinically as statins act by mobilizing bone-marrow-derived EPC via the Akt pathway, resulting in increased numbers of EPC with enhanced functional activity and migration capacity.

Non-pharmacologic interventions such as physical exercise have been reported to significantly increase number and function of circulating EPC in patients who resumed a standardized physical activity during a rehabilitation programme, in patients with coronary artery disease (CAD) and PAD, and in healthy individuals exercising for ≥30 min, possibly via a VEGF-dependent mechanism. Patients with CAD have a number of identifiable risk factors which have an inverse correlation with EPC senescence. This relationship illustrates the role of EPC in cardiovascular pathophysiology, and highlights the potential of EPC as adjuvant biomarkers in arterial disease, but the problems with characterization and identification of EPC need to be resolved before this potential can be realized in a clinical setting.

**Role of EPC in Myocardial Ischaemia**

The majority of work investigating the role of EPC has been related to myocardial disease, including acute myocardial infarction (AMI), CAD, chronic left ventricular dysfunction and ischaemic cardiomyopathy. To date, over 20 randomized human trials investigating the role of EPC in myocardial disease have been published, with a number of trials currently actively recruiting. However, meta-analysis of these trials is not feasible because of varying protocols, dose regimens, patient populations and randomization criteria used.
The TOPCARE-AMI trial was one of the initial pilot trials investigating the potential of progenitor cell therapy in AMI. 29 19 patients with reperfused AMI were randomly assigned to receive an intracoronary (into the infarcted artery) infusion of either autologous BMDC or circulating-blood-derived progenitor cells into the infarct artery with 11 patients in the control group. Best medical therapy was given to all patients. At 4 months an increase in left ventricular ejection fraction (LVEF), improved regional wall motion in the infarct zone, reduced end systolic left ventricular volumes and increased myocardial viability in the infarct zone were reported, compared with the control group. No differences were detected between the peripheral blood-derived progenitor cells and BMDC groups and no adverse reactions were reported. 29 Although this trial established feasibility of progenitor cell therapy, it did not establish the beneficial role of any one specific cell lineage. It is not certain that endothelial cell incorporation leading to neoangiogenesis was responsible for the improvement noted. 25% of the patients in the cell therapy group had re-stenosis of the stented lesion although new collaterals at these sites were observed and no comparative information is available for the control group. This trial eventually recruited 59 patients in the progenitor cell therapy group and has recently reported sustained improvement in cardiac function at 5 years with no long-term side effects. 30

The BOOST study investigated the effect of an injection of autologous unfractionated BMDC in patients post MI. 31 60 patients were randomized to treatment versus control (optimum post infarction medical treatment only) with 30 in each group. At 6 months it was demonstrated that the LVEF was 6% higher in the BMDC-infused patients than in the control group and there was associated improvement in systolic wall motion. However these improvements were not sustained at 18 months.

The largest and most recent trial to date is the REPAIR-AMI that investigated the effect of infusion of basement-membrane-derived mononuclear cells post-AMI. 32 199 patients were recruited to the trial and allocated by double blind randomization to receive mononuclear cells or placebo. 101 patients received infusion of mononuclear cells; 98 patients received placebo. At 4 months the treatment group demonstrated a moderate improvement in LVEF, (5.5% versus 3% in the control group, \( p = 0.01 \)) which was sustained at one year.

The role of EPC in relation to cardiomyopathy and chronic left ventricular dysfunction has also been investigated. Two small published trials (23 patients and 27 patients) have investigated the effect of an infusion of autologous mononuclear cells on patients with ischaemic cardiomyopathy. 33 Both of these trials are non-randomized and give limited evidence relating to the potential benefit of progenitor cells in heart failure, but they do suggest an improved treadmill performance following injection. The ‘Progenitor Cell Therapy in Dilative Cardiomyopathy’ study is currently recruiting to establish the effect of an intracoronary infusion of bone-marrow-derived mononuclear cells (BMMNC) on cardiovascular function in patients with established cardiomyopathy. 34

The TOPCARE-CHD registry, 35,36 involving 121 consecutive patients with an MI in the previous 3 months and pooled results from the TOPCARE-CHD pilot and crossover trial, showed that N-terminal pro-atrial natriuretic peptide (NT-proANP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) serum levels were reduced 3 months after transcoronary administration of BMDC. In this study, colony-forming capacity was used as a measure of the functional capacity of the administered cells as described previously. The results also demonstrated that patients receiving a higher proportion of colony-forming cells had significantly lower cardiac mortality rates than those receiving a lower proportion. 36

A generous body of translational evidence now exists supporting the potential of progenitor cell therapy in cardiology. The most common method of progenitor cell procurement appears to be autologous transplantation of ex vivo expanded BMMNC. This consists of a heterogenous suspension of progenitor cells with varying proportions of CD34/CD45/CD133 positive cells. It has not been possible to decide the exact mechanisms responsible for the observed benefits with this therapy – direct cellular effect or paracrine effect. Some protocols also include G-CSF pre-treatment for more robust and viable progenitor cell harvest as these agents facilitate mobilization of HSC and EPC into the peripheral blood. 37 These agents are pro-coagulant and pro-inflammatory and can induce angina themselves. Since these agents have a strong pro-angiogenic affect of their own, via mediation of VEGF and similar peptides, the beneficial affect attributed to EPC/progenitor cells may actually be due to the paracrine affect of G-CSF.

The Role of EPC in Peripheral Ischaemia

Extrapolation of evidence from animal studies to human studies relating to the feasibility of autologous progenitor cell transplantation in limb ischaemia is limited. In the ‘Therapeutic Angiogenesis using Cell Transplantation’ (TACT) study (2002), 38 115 patients (74 limbs PAD and 41 limbs thromboangiitis obliterans) were randomly assigned to injection of autologous BMMNC or placebo PBMNC into the gastrocnemius muscle of the affected limb. At 4 weeks, those legs into which BMNC had been injected had improved ankle-brachial pressure index (ABPI), improved transcutaneous oxygen pressure, reduced rest pain and increased pain-free walking time. These improvements were sustained at 24 weeks without serious complications.

Recently the long-term results of this trial have been published. 39 These demonstrated a 3-year amputation-free rate of 60% (95% CI 46–74) in PAD (Fontaine stage III and IV) and 91% (95% CI 82–100) in patients with thromboangiitis obliterans in the intervention limb. ABPI did not change significantly during the 2-year follow-up period in either group of patients. Pain scale and ulcer size were, however, improved significantly (\( p < 0.001 \)). Similar to cardiac studies, delineation of the specific mechanism of effect is lacking; improvement is attributed to the neoangiogenesis promoted by the BMMNC which contain EPC fractions and their associated paracrine effect. These factors possibly augment the local angiogenic effect of ischaemia itself but evidence of transdifferentiation of progenitor cell lineages is lacking. It is also difficult to interpret these results as no control data are presented and therefore no conclusion can be made regarding long-term comparative effects of treatment.
Prior to the long-term report of the TACT study, Higashi et al. and Saigawa et al. reported encouraging results using similar protocols in their randomized studies including small numbers of patients. Lenk et al. were able to achieve a similar beneficial effect with intra-arterial infusion of PBMC (harvested following priming with G-CSF) in 7 patients with infrapopliteal PAD and critical ischaemia. They reported no side effects with G-CSF stimulation of intra-arterial infusion and noted a thirty-fold improvement in pain-free walking distance and a concomitant improvement in ABPI at 12 weeks. There were no controls studied in this group.

Ishida et al. conducted a similar study using G-CSF therapy for 5 days prior to PBMC including CD34+ cell harvesting and intramuscular injection into the ischaemic limbs. This was a small study in 6 patients (5 thromboangiitis obliterans and 1 critical ischaemia) but some encouraging results have been reported. ABPI improvement associated with a 300% increase in pain-free walking and 200% increase in total walking distance was noted at 4 weeks post-therapy and results were sustained at 6 months. A variable degree of pain relief was achieved at 10 days but, more importantly, ulcer healing was noted in all three patients with ulcers. This group has reported cell therapy as an alternative to angioplasty but given the nature of this study it would be optimistic at this stage. However, they were able to repeat the treatment in 2 patients where ulcers recurred at 1 year. Again, it is unjustified to pin down the benefit to EPC alone. Similar results following PBMC transplant post-G-CSF priming into 92 ischaemic limbs has been reported by Kawamura et al. in a slightly different population of PAD. 68 of the 92 patients treated were on haemodialysis for chronic renal failure. But some of the patients in Fontaine I and II classes have also been classified as critically ischaemic in this study. The total amputation rate was very high (64%) in Fontaine IV group (53/92), which is the group likely to gain most benefit from such treatment.

While most of the studies have reported favourable results, some important insights can be gained from the study reported by Miyamoto et al. BMNC harvested from the iliac crest were injected in 11 ischaemic limbs of 8 patients with thromboangiitis obliterans. No G-CSF priming was done. Initial improvement on a visual analogue scale was noted in all patients and ulcers healed completely in 88% cases. However, 50% of cases experienced adverse affects on follow-up: 1 case of sudden death of unknown origin in a 30-year-old without any previous cardiac history at 20 months, AV fistula formation at 7 months in 1 patient, and 2 patients with worsening symptoms. The initial report of the TACT study also reported 2 sudden deaths out of 25 people treated, within 24 weeks, but the population in the TACT study was much older compared to this study. An association has also been noted between unstable angina and the intra-plaque presence of angiogenic peptides in animal studies; it has therefore been suggested that these agents might play a role in plaque progression and instability. The biological activity of most of the angiogenesis agents currently being tested clinically are highly potent, and it is likely that this could cause unwanted neo-vascularization in non-targeted tissues such as in patients with diabetic retinopathy who have been found to have increased numbers of receptors for VEGF. There are several current trials, the results of which are awaited, but clearly the importance of long-term follow-up in patients undergoing cell therapy cannot be underestimated.

**Role of EPC in Cerebrovascular Disease**

The role of EPC in cerebrovascular ischaemia is currently under investigation but there a paucity of data from human studies. The vast majority of studies on the effects of EPC on outcomes after cerebral ischaemia have been conducted in mice and in ischaemic stroke following medium and large vessel disease. While these studies have shown positive outcomes, there is currently no consensus on which EPC cell type is optimal or the optimal timing for the administration of stem cell therapies post-stroke. However, EPC administration has been shown to improve clinical outcomes after an ischaemic stroke in animal models and EPC levels have been shown to be of prognostic value in human studies. Low levels of circulating CD34+ EPC are strongly predictive of severe neurological impairment (day 2) and adverse clinical outcomes (day 90) following an ischaemic stroke.

The exact mechanism of action of EPC in the brain is poorly understood. There is some evidence that PBMC are capable of differentiating into neural cells and are thus theoretically capable of structural regeneration but most in vivo studies of peripheral or cord-blood-derived EPC demonstrate relatively few cells incorporated into ischaemic tissue. The disproportionately large improvement in clinical outcome in mice treated with stem cell therapies post-stroke compared to the actual number of stem cells incorporated into ischaemic brain tissue indicate that the restorative effect of these therapies is not entirely due to transplanted cells. It is most likely the paracrine affect of EPC which results in the reorganisation of nerve fibres in the injured brain. This augments neuroplasticity and thus improves long-term clinical outcomes in treated animals.

**Conclusion**

The holy grail of stem cell research is to develop the capability of isolating specific progenitors from a donor source, which can then be expanded ex vivo prior to transplantation, within the time-frame of an emergent situation such as a stroke, myocardial infarction or acute/critical limb ischaemia, where the benefit achieved is most likely to be greatest. But before this stage is reached there are some critical issues which hinder the translation of cell therapeutics. One of the problems is lack of standardization in classification and characterization of donor cells and our inability to track migration and differentiation of these cells in vivo after an intervention has been carried out. This has serious implications for quality control of transplantable cells and indeed for the safety of clinical trials. There is a definite gap between what is known about specific EPC subpopulations from experimental in vitro studies and clinical trials, which use largely unfractioned BMNC or PBMC samples because of the scarcity of EPC in any one anatomical niche. This leads to difficulties in determining the mechanism of action of these cell therapies in clinical practice and the relative efficacy of EPC subpopulations.
alone. Furthermore, current evidence suggests that the paracrine effect of growth factors secreted by these cells plays a more significant role than previously thought. With our present level of understanding it is not possible to refute the suggestion that cell therapies may merely be augmenting hypoxia- or ischaemia-induced angiogenesis.

To date the evidence of the efficacy of EPC administration in PAD is limited and the isolated impact of EPC remains undetermined. PBMCN or BMMNC therapies seem to have a beneficial effect in the short term, but long-term efficacy and safety is still undetermined. Trials so far have largely been limited to small uncontrolled or poorly randomized studies. Although a beneficial effect is observed, the mechanism of this effect seems to be different from the one postulated by animal and in vitro studies. There is an imminent need to set up a regulatory body to control the quality of future trials by standardizing dosage, route and type of cells to be transplanted, and all patients should have a long-term follow-up for safety issues as mechanisms for regulating uncontrolled cell division once injected are not available. Specific trials are also required for G-CSF therapy versus G-CSF-primed EPC therapy before the beneficial effect can be attributed to EPC. This paradigm will take some time to resolve and until then clinicians should view the available data with an open but critical mind.

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