BIOCELL
2022 46(7): 1593-1598

Tech Science Press

Prognostic, diagnostic and therapeutic potential of endothelial progenitor cells for patients with ischaemic stroke: Hype or Hope

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Key words: Ischaemic stroke, Endothelial Progenitor Cells, Stroke therapeutics, Clinical biomarkers, Regenerative medicine

Abstract: Ischaemic stroke is a debilitating disease with immense personal, societal and economic impact. Thrombolysis with recombinant tissue plasminogen activator remains the only approved pharmacotherapy for this disease. As each year less than 1% of eligible patients receive this therapy worldwide, efficacious new therapeutics are desperately needed. Emerging evidence suggest endothelial progenitor cells (EPCs), capable of repairing damaged vasculature, as one such therapeutics. However, questions regarding their optimal dose, delivery route and *in vivo* survivability remain largely unanswered. Outgrowth endothelial cells, generated in large numbers by *ex vivo* expansion of EPCs, enable effective assessment of these issues and may eventually serve as off-the-shelf therapeutics. Correlations between circulating EPC levels and stroke outcome imply that EPCs may also serve as clinical biomarkers for stroke. This viewpoint briefly evaluates the current evidence, pinpoints the gaps in the literature and proposes new directions for research.

Abbreviations

BBB:	Blood-brain barrier
BMSC:	Bone marrow-derived mononuclear stem cell
EPC:	Endothelial progenitor cell
HLA:	Human leukocyte antigen
IS:	Ischaemic stroke
OEC:	Outgrowth endothelial cell
PDGF-BB:	Platelet-derived growth factor-BB
RACs:	Regeneration-associated cells
rtPA:	recombinant tissue plasminogen activator
TNF-α:	Tumour necrosis factor-α
VEGF:	Vascular endothelial growth factor

Introduction

Stroke annually affects about 13.5 million people worldwide and constitutes one of the leading causes of mortality and morbidity (Virani *et al.*, 2021). Ischaemic stroke, stemming from the occlusion of an artery leading to the brain, represents the major subtype of stroke and accounts for approximately 85% of all strokes in the Western world (Hisham and Bayraktutan, 2013). At present, reperfusion therapy with recombinant tissue plasminogen activator (rtPA) and mechanical thrombectomy are the only approved treatment options for acute IS. However, due to short therapeutic windows associated with these treatment regimens, each year globally <1% of patients receive these therapies (Malhotra *et al.*, 2019; Virani *et al.*, 2021).

The pathophysiology of stroke is complex and involves many interrelated processes, including depletion of ATP, apoptosis, necrosis, excitotoxicity, inflammation, oxidative stress and an abrupt surge in intracellular calcium levels (Allen and Bayraktutan, 2008; Rakkar and Bayraktutan, 2016). Despite having tremendous success in preclinical studies, agents targeting these particular mechanisms have failed to produce similar benefits in clinical trials. The incessant failure of clinical trials with the so-called neuroprotectants prompted search for alternative therapeutics and placed cell-based therapeutic approaches targeting cerebrovascular integrity at the forefront of clinical investigation. Given that disruption of blood-brain barrier (BBB) and ensuing cerebral oedema constitute the main cause of death within the first week after an IS, adoption of a vascular restorative/reparative approach was somewhat inevitable (Hou and MacManus, 2002; Dankbaar *et al.*, 2011). In this context, endothelial progenitor cells (EPCs), capable of angiogenesis, vasculogenesis, self-renewal and differentiation into mature endothelial cells, have attracted much of the attention (Asahara *et al.*, 1999; Bayraktutan, 2017; Bayraktutan, 2019).

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Received: 10 August 2021; Accepted: 23 August 2021

Doi: 10.32604/biocell.2022.018679

Overview of EPCs as Therapeutics

Phase I studies, performed with bone marrow-derived mononuclear stem cells (BMSCs) or CD34+ cells known to contain a population of progenitor cells, prove the safety and feasibility of intravenous and intra-arterial infusion of EPCs during the (sub)acute phase of IS (Fang *et al.*, 2019; Savitz *et al.*, 2011). Despite confirming these findings, a randomised, multicentre study using BMSCs yielded no benefit on stroke outcome (Prasad *et al.*, 2014). Preclinical studies, investigating the degree of EPC incorporation into the damaged vasculature as a measure of therapeutic efficacy, also report inconsistent results in terms of functional outcome (Purhonen *et al.*, 2008; Garbuzova-Davis *et al.*, 2017; Hong *et al.*, 2020). Indeed, while robust engraftment of intravenously administered human bone marrow EPCs within capillaries is associated with widespread repair of vasculature and near normal morphology of endothelial cells, astrocytes and pericytes in bilateral striatum and motor cortex of a rodent model of transient IS (Garbuzova-Davis *et al.*, 2017), studies showing inability of circulating EPCs to engraft vasculature also exists (Hagensen *et al.*, 2012). As ambiguities regarding the nature of EPCs somewhat account for these discrepancies, it is of utmost importance to standardise the definition of EPCs in order to accurately evaluate their therapeutic efficacy. As alluded above, CD34+ cells, isolated from peripheral blood by fluorescence activated cell sorting, are described in various studies as EPCs (Banerjee *et al.*, 2014; Shyu *et al.*, 2006). Although shown to promote revascularisation in ischaemic hearts and brains, it is unlikely that these haematopoietic stem cells can actually be true EPCs (Chen *et al.*, 2014; Sobrino *et al.*, 2011). Because, EPCs possess embryonic angioblast-like characteristics and are the precursors of mature endothelial cells, it is likely that only few circulating nonhaematopoietic cells (e.g., CD45- or CD14-), concomitantly expressing markers for stemness (e.g., CD34+ or CD117+), immaturity (e.g., CD133+) and endothelial cells (e.g., KDR+ or CD31+), may make up the true EPCs. It is of note that a recent study suggests ephrinB2 and bone morphogenetic protein 2 and 4 as important novel markers for identification and characterisation of EPC subpopulation in adult peripheral blood (Abdelgawad *et al.*, 2021).

In addition to repairing vascular damage, EPCs can also stimulate the process of endogenous recovery by immunomodulation and secretion of various trophic factors, notably stromal-derived factor-1, angiopoietin-1, vascular endothelial growth factor (VEGF), basic fibroblast growth factor and platelet-derived growth factor-BB (Ohab *et al.*, 2006; Rosell *et al.*, 2013). Enhanced functional recovery coupled with decreases in infarct volume, angiogenesis and anti-inflammatory cytokine release provide indirect evidence for the beneficial effects of stem cells (and their secretome) in animal models of stroke (di Santo *et al.*, 2009; Rosell *et al.*, 2013). Our recent studies probing how EPCs, defined as CD45-CD34+CD133+KDR+ cells, may affect the integrity and function of an *in vitro* model of human BBB subjected to ischaemic injury or TNF-α, a key cytokine during the post-stroke inflammation, offers convincing direct evidence for their protective effects in that suppression of stress fibre formation, oxidative stress and apoptosis play a pivotal role (Abdulkadir *et al.*, 2020; Alwjwaj *et al.*, 2021). Crucially, these studies also indicate that much of the EPC-mediated beneficial effects are realised by their secretome and thus necessitate the scrutiny of EPC secretome as a potential therapeutic for IS. Microparticles, containing DNA, RNA and microRNAs, represent an important constituent of the secretome and deserve attention in future studies due to their seminal role in inducing vascular endothelial regeneration *via* promotion of tissue-resident endothelial cell proliferation and migration (Kadir *et al.*, 2020). Concurrent manipulation of other key factors known to mediate mobilisation, homing and differentiation of EPCs, such as VEGF, stromal derived factor-1 and interleukin-10 also deserve attention in relevant future studies (Bayraktutan, 2019; Nagata *et al.*, 2019).

EPCs as Clinical Biomarkers

Since EPCs repair the damaged vasculature and can also work as proangiogenic support cells, the number of circulating EPCs is widely considered as a reliable diagnostic and/or prognostic marker for IS. Indeed, observational studies scrutinising the correlation between EPC number and stroke outcome have correlated the increased cell numbers in acute and subacute phases of the disease to greater vascular repair, endothelial restoration and better clinical outcome (Paczkowska *et al.*, 2009; Martí-Fàbregas *et al.*, 2013). However, investigations reporting lower baseline levels of circulating EPCs in acute IS patients compared to healthy volunteers also exist (Tsai *et al.*, 2014). In addition to EPC number, variations in the capacity of cultured EPCs to migrate, proliferate and form tubules and colonies are also regarded as important diagnostic and prognostic markers for IS. Even so, the current data on these EPC characteristics during different phases of stroke, particularly chronic phase, are rather scant and controversial. Besides, exclusion of healthy volunteers from most studies make it difficult to interpret the data generated (Chu *et al.*, 2008; Martí-Fàbregas *et al.*, 2013; Zhou *et al.*, 2009). Considering the gap in the literature and bearing in mind the close association reported between EPC numbers and different subtypes of stroke (Tsai *et al.*, 2014), a recent study has longitudinally assessed the diagnostic and prognostic value of EPCs in elderly patients with lacunar or cortical stroke during acute, subacute and chronic phases of the disease (Rakkar *et al.*, 2020). By recruiting both elderly (≥65 years) and young (18–64 years) healthy volunteers, this study has also addressed the specific association between EPC characteristics (number and functional aspects) and chronological ageing, a prominent risk factor for IS. The data indicate that ageing process and other vascular risk factors, including hypertension and diabetes, adversely affect the release and function of EPCs in healthy volunteers and the count and function of EPCs are similar in lacunar and cortical stroke patients during different phases of the disease.

OEC Therapy for Stroke

Cell culture, based on adhesion of cells to specific substrates, e.g., collagen, before culture in endothelial cell specific media is recognised as the best methodology to generate

adequate numbers of homogeneous cells for therapeutic purposes. This procedure leads to generation of two functionally and morphologically distinct EPC subtypes: early EPCs (eEPCs) and outgrowth endothelial cells (OECs), also known as endothelial colony forming cells (Bayraktutan, 2019). The molecular profiling of early EPCs classifies them as haematopoietic cells (Medina *et al.*, 2010a). OECs, on the other hand, constitute the functional subtype of EPCs and display strong proliferative, migratory and tubulogenic capacity *in vitro*. OECs also express endothelial and progenitor cell markers which differentiate them from haematopoietic cells and circulating mature endothelial cells, respectively (Abdulkadir *et al.*, 2020). Attempts to expand OECs *ex vivo* have shown that after a certain number of passages, OECs go into senescence and display classical markers of senescence including enlarged cellular phenotype, S-β-galactosidase activity and DNA damage. Albeit associated with dysfunctionality and limited regenerative potential, OEC senescence is also associated with the reduced risk of tumourigenesis, indicating their safety *in vivo* (Medina *et al.*, 2013). Ability of OECs to lodge and survive in nine different vascular beds for up to 7 months after injection without inducing any thrombosis or infarcts confirm their long-term efficacy and safety (Milbauer *et al.*, 2009). Through modulation of key mechanisms involved in OEC senescence, such as interleukin-8 and growth differentiation function 15, it is possible to delay senescence in a controlled manner and/or improve vascular functionality (Medina *et al.*, 2013; Ha *et al.*, 2019).

Key features that make OECs potentially a very effective cell-based therapeutic include that OECs repair post-ischaemic vascular injuries by directly incorporating into host endothelium and inducing angiogenesis. Significant decreases in avascular areas in a murine model of retinal ischemia and improvements in cardiac function in a porcine model of acute myocardial infarction support this hypothesis (Medina *et al.*, 2010b; Dubois *et al.*, 2010). Other features include that OECs can be expanded from patients' own blood for autologous therapy which markedly eliminate the risk of rejection. However, the time needed to cultivate the number of cells required for transplantation restricts autologous therapy to chronic phases of disease and necessitates consideration of an allogeneic approach for patients with acute and subacute stroke. Albeit conceptually fraught with immunological risk, a meta-analysis of large animals with ischaemic heart disease has shown that both autologous and allogeneic cell therapies are equally safe and efficacious (Jansen *et al.*, 2015). Detection of a greater tissue repair in acute ischaemic stroke patients at one year of receiving intravenously injected allogeneic cells further confirm the safety of this therapeutic approach (Vaes *et al.*, 2012) where suppression of both CD4 and CD8 T cells proliferation and activation by EPCs may be crucial (Naserian *et al.*, 2020). It may be possible to further augment the immunosuppressive capacity of EPCs by priming them with TNF-α to specifically activate TNF/TNFR2 (TNF-α receptor 2) signalling pathway before administration (Barkestani *et al.*, 2021). Creation of cell banks with detailed characterisation of HLA haplotypes matching population requirements and use of immunologically immature cord blood-OECs may also help address immune-(in)compatibility issue. By mediating the secretion of both interleukin-10, an anti-inflammatory cytokine and transforming growth factor-β1, (TGF-β1), cord blood-OECs restore a functional vascular network under ischaemic conditions in immunocompetent mice (Proust *et al.*, 2020). TGF-β1 is a multifunctional protein that plays a crucial role in the formation of blood vessels, wound healing and inflammatory processes in the immune system (Fujio *et al.*, 2016).

Administration of regeneration-associated cells (RACs) may be an alternative approach to potentiate the therapeutic efficacy of EPCs in ischaemic diseases. RACs, obtained by vasculogenic conditioning of peripheral mononuclear cells in the presence of human recombinant stem cell factor, thrombopoietin, Flt-3 ligand, VEGF and interleukin-6, can enhance EPC expansion and activate anti-inflammatory and angiogenic monocytes/macrophages and helper T lymphocytes (Masuda *et al.*, 2012; Masuda *et al.*, 2014). In accordance with these findings, administration of RACs to an animal model of human transient IS during the acute phase of ischaemic injury has successfully reduced infarct volume and promoted significant recovery of neural tissues through intensified angiogenic and anti-inflammatory effects (Nakayama *et al.*, 2019).

Future Issues for Consideration

As summarised in Fig. 1, due to extremely low level of true EPCs in circulation, it is highly unlikely to sort sufficient number of cells from peripheral blood that can be used for therapeutic purposes. OECs, the functional subtype of EPCs generated by cell culture, appear to be a highly promising therapeutics with immense potential for post-ischaemic vascular repair, vasculogenesis, angiogenesis and possibly neurogenesis (Takizawa *et al.*, 2016, Bayraktutan, 2019). Although limited replicative potential and the current immunological understanding, based on animal and clinical data, support the use of allogeneic therapy, various issues concerning the survivability, *in vivo* tracking, optimal dose and delivery route need clarification. Furthermore, questions regarding tolerance to repeat OEC dosing need to be addressed. Should cells from a different donor be used to avoid anamnestic reaction? Also, questions regarding the co-application of OECs with other stem cells or agents targeting oxidative stress, inflammation or increased intracellular Ca^{2+} levels need to be addressed. How would these applications affect the therapeutic impact of OECs? Similarly, questions regarding immunological reactions also need to be addressed. Should HLA-matching be performed before administration of OECs? Should immunological reaction alongside potential (serious) adverse effects be monitored after administration?

In conclusion, well-planned comprehensive studies, monitoring the levels and functional capacity of circulating EPCs and the immunologic profile of the recipients, are needed to unravel the true diagnostic or therapeutic value of EPCs/OECs for IS patients.

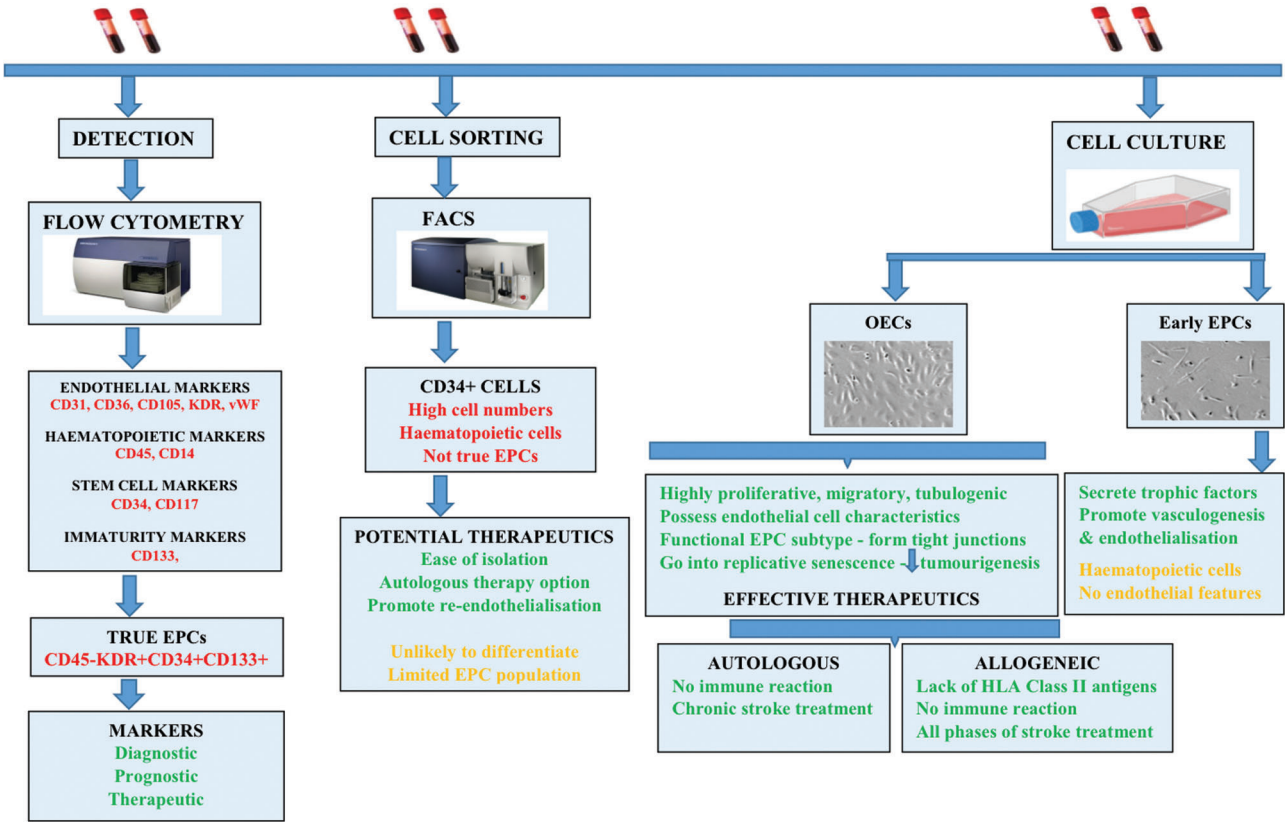

FIGURE 1. Summary of the main processes by which endothelial progenitor cells (EPCs) are obtained from peripheral blood and used for clinical purposes. EPCs can serve as diagnostic, prognostic or therapeutic biomarkers for ischaemic stroke. Alternatively, EPCs may be sorted by FACS or expanded by cell culture to obtain a large number of homogenous cells which successively yield CD34+ cells and outgrowth endothelial cells (OECs). These then can be used as efficacious therapeutics in autologous or allogeneic therapies.

Acknowledgement: Funders are thanked for support of this writing.

Availability of Data and Materials: No data are included in this viewpoint.

Author Contribution: The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

Ethics Approval: No committees were required for this study.

Funding Statement: This work was supported by a grant to Dr. Ulvi Bayraktutan from the Dunhill Medical Trust (R459/0216). The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The contents are solely the responsibility of the author and do not necessarily represent the official views of the DMT.

Conflicts of Interest: The author declares that they have no conflicts of interest to report regarding the present study.

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