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Endothelial Progenitor Cells: potential novel therapeutics for ischaemic stroke

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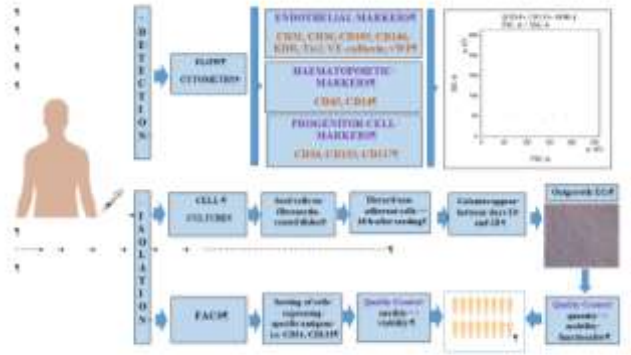
Graphical abstract



ABSTRACT

Stroke is classified into two main groups depending on its aetiology; ischaemic stroke and haemorrhagic stroke which successively develop from the occlusion or rupture of an artery leading to the brain. Despite being the leading cause of human cerebral damage, there is currently no medical therapy for haemorrhagic stroke and thrombolysis with recombinant tissue plasminogen activator remains the only approved pharmacotherapy for ischaemic stroke. However, due to its short therapeutic window (first 4.5 h of stroke onset) and increased risk of haemorrhage beyond this point, globally each year less than 1% of patients receive this therapy. Since, endothelial dysfunction, associated with inflammation and vascular permeability, remains the key early event in the pathogenesis of stroke, endogenous element(s) capable of countering this defect may help maintain vascular homeostasis and explain the overt differences observed in patients' functional outcome. Accumulating evidence indicate that bone marrow-derived endothelial progenitor cells (EPCs) equipped with an inherent capacity to repair endothelial damage and differentiate into few other cell lines represent one such element. Indeed, EPC-based cell therapy, backed by rigorous preclinical, translational and early proof-of-concept, safety and feasibility clinical studies, is now considered as an important novel therapeutic approach. However, several questions relating to optimal cell dosage, delivery route and immediate and sufficient availability of cells remain to be addressed before its efficacious translation to clinical practice. In this context, *ex vivo* expansion

of EPCs leading to an abundant generation of functional outgrowth endothelial cells offers a great opportunity to address these issues and create a novel off-the-shelf type of therapeutic product.

1. Introduction

Stroke continues to be one of the leading causes of mortality and morbidity in the World. Each year, globally more than 15 million people suffer a first stroke, of these one-third die and another one-third are left permanently disabled [1, 2]. In the United Kingdom, about 100,000 people suffer a stroke each year, equating to one person every 5 minutes and there are over 1 million people living with mild to severe disability caused by stroke [3]. Although everyone, including children, are at risk of having a stroke, more than two third of the people affected are older than 65 years of age [4]. In addition to human suffering, the financial costs of stroke covering both direct (diagnosis and inpatient/outpatient care) and indirect (social benefit payments to patients, income loss, carer costs for home nursing) expenses are also very high and amount to ~£9 billion per year in the UK alone [5].

There are two main types of stroke, haemorrhagic and ischaemic. Haemorrhagic strokes stem from the leak or rupture of an artery within (intracerebral haemorrhage, ICH) or on the surface (subarachnoid haemorrhage, SAH) of the brain tissue. They constitute about 15% of all strokes in the Western World but account for much of the stroke-related mortalities [6]. Ischaemic strokes, on the other hand, make up about 85% of all strokes and derive from the occlusion of a blood vessel leading to or within the brain due to formation of an embolus (embolic strokes) or a thrombus (thrombotic strokes). Ischaemic strokes are further stratified into several subgroups due to aetiological differences, namely small-vessel disease, large-artery atherosclerosis, cardioembolic stroke and cryptogenic stroke [7].

A large number of modifiable and non-modifiable risk factors are associated with an elevated risk of stroke. While the modifiable risk factors include those emerging from life style choices and the environment, the non-modifiable risk factors encompass factors related to hereditary or natural

processes [8-10]. Figure 1 summarises some of these risk factors for stroke and the physiological functions compromised as a result.

Despite being the leading cause of human brain damage, there is currently no medical therapy for haemorrhagic stroke and thrombolysis with recombinant tissue plasminogen activator (rt-PA) remains the only approved pharmacotherapy for ischaemic stroke. However, given the short therapeutic window (within 4.5 h of stroke onset) and markedly enhanced risk of haemorrhage beyond this point, globally less than 1% of patients receive this therapy each year [11]. New therapeutic strategies including application of novel fibrinolytics (e.g. desmetoplase and tenecteplase), glycoprotein IIb/IIIa antagonists (e.g. tirofiban and abciximab) and interventional approaches comprising both mechanical thrombectomy (clot retrieval or suction) and disruption (intracranial angioplasty) may set to enhance the number of patients receiving reperfusive treatment [12].

Naturally, the limited availability of therapeutic options coupled with higher mortality and morbidity have spurred stroke research community over the years to discover safer and more efficacious novel treatment regimens for stroke patients, particularly for those who are unsuitable for thrombolytic or interventional therapies. Intriguingly, despite showing tremendous success in experimental studies, none of the so-called therapeutic agents replicated the favourable effects in subsequent clinical trials. Reasons for such a fundamental failure are multifactorial and include mostly targeting specific pathways pertaining to recanalisation or excitotoxicity and the use of mainly young and healthy male animals subjected to middle cerebral artery occlusion (MCAo) in preclinical studies [13]. In contrast, stroke patients often suffer from various comorbidities like a prior ischaemic attack, display patho-clinical heterogeneity in relation to aetiology or affected arteries and routinely take several medications for various cardiovascular risk factors, notably hypertension and diabetes. Besides, to judge the efficacy of a given therapeutic compound, clinical trials assess the long-term neurological and functional parameters (≥90 day of stroke onset) while preclinical studies focus more on infarct or oedema volumes during early stages of stroke [14].

Given that cerebrovascular recovery accompanied by angiogenesis, neurogenesis and re-endothelialisation is fast emerging as a powerful new concept for stroke therapy and also that many processes, other than excitotoxicity, such as excessive release of reactive oxygen species, increased availability of intracellular Ca^{2+}, mitochondrial dysfunction, inflammation and apoptosis are spatially and temporally involved in the pathophysiology of stroke, it is important to discover and test new mediators that can simultaneously target several mechanisms, demonstrate long-term effects and can be used beyond the initial phases of stroke [15-21].

2. Endothelium and endothelial damage in stroke

Endothelium covers the entire inner surface of all blood vessels whereby constitutes a multifunctional organ tasked with the regulation of vascular tone, thrombosis, angiogenesis, inflammatory status and the selective passage of molecules between peripheral circulation and the surrounding tissue. Endothelial dysfunction is deemed to exist when normal endothelial function can no longer be sustained either in the basal state or in response to any physical (e.g. flow), humoral (e.g. bradykinin), chemical (e.g. acetylcholine) or pathological (e.g. hypertension) stimuli [22, 23].

Endothelial dysfunction is consistently observed in patients with stroke and may be regarded as the key early event in development of arteriosclerotic disease and associated complications such as thrombus formation and plaque disruption. Besides, by promoting peripheral vascular resistance and thus accentuating hypertensive state, endothelial dysfunction also worsens neurological and functional deficits in stroke patients [23, 24]. Even in cases where endothelial dysfunction is not regarded as the primary insult, much of the secondary events affecting the overall severity and outcome of stroke appear to be of endothelial origin. Delayed cerebral ischaemia or vasospasm following SAH and coagulopathies, aneurysms or amyloid angiopathy preceding ICH are important examples for such secondary events [25, 26]. Furthermore, endothelial dysfunction also constitutes the first step in blood-brain barrier (BBB) dysfunction which is characterised by

disruption of the endothelial integrity and leakage of blood constituents into the brain parenchyma [27]. Since formation of brain oedema (specifically focal brain oedema after ischaemic stroke, global brain oedema after SAH and perihaematomal oedema after ICH) constitute the main cause of death or neurological deterioration after stroke, restoration of endothelial integrity and BBB function may be a very effective therapeutic strategy to mitigate stroke-related damage [25, 26]. Although endothelial maintenance after vessel injury is in part mediated by division and lateral migration of neighbouring endothelial cells, endothelial progenitor cells (EPCs) are thought to significantly contribute to this mending process [28].

3. Definition and identification of EPCs

EPCs are described as a population of new cells released into peripheral blood by bone marrow to promote endothelial repair and neovasculogenesis in response to an ischaemic injury [29]. Flow cytometry, based on the multi-parameter analysis of single cells in a heterogeneous cell population through concurrent detection of various cell surface markers, is one of the most commonly used methodologies to identify EPCs in whole blood. However, the significant overlap of markers expressed on the surface of EPCs and haematopoietic cells makes it hard to accurately identify EPCs through this methodology [29-31]. Considering that EPCs possess embryonic angioblast-like characteristics and are committed to differentiate into mature endothelial cells, employment of a panel of antibodies targeting markers for haematopoietic cells (CD45), immaturity (CD133), stemness (CD34) and endothelial maturity (KDR) may be the best option to detect all endothelial-committed (CD34+CD133+KDR+CD45-, CD34+KDR+CD45- and CD133+KDR+CD45-) and undifferentiated (CD34+CD133+CD45-, CD34+CD45- and CD133+CD45-) EPCs (Fig. 2).

Fluorescence-activated cell sorting (FACS) is also frequently used to isolate EPCs (mainly CD34 expressing cells) from peripheral blood or bone marrow to permit their use in clinical trials. Although shown to promote revascularisation in various ischaemic events affecting the heart or

the brain, these CD34+ cell populations are likely to contain a large quantities of haematopoietic stem cells and mature endothelial cells rather than the actual progenitor cells [32, 33].

4. EPCs as potential biomarkers

The resolution of oedema and neuro-inflammation that develop immediately after an ischaemic injury may in part explain the early functional recovery observed following stroke. However, considering that stroke is a non-progressive localised cerebral condition and patients with stroke go on to manifest different levels of disability or no disability at all, endogenous components such as EPCs may be of pivotal importance to repair neurovascular damage and help determine the extent of functional recovery, implying that variations in the level and functional capacity of EPCs may serve as clinical markers [34, 35]. Indeed, increasing number of preclinical and clinical studies have reported significant correlations between circulating EPC levels and the severity or outcome of various cardiovascular, malignant, metabolic or inflammatory disorders [36, 37]. However, the data on the extent and time course of EPC release in experimental and clinical ischaemic stroke remain limited and inconsistent. For example, while a significant increase in CD34+CD133+ EPC numbers has been reported within the first 24 hour of an acute ischaemic attack by Paczkowska et al, others have shown initially lower but steadily increasing numbers of CD133+KDR+ cells in similar patient groups compared to the healthy subjects. In the latter studies, the numbers of CD133+KDR+ were shown to peak at day 7 where higher numbers correlated to better outcome in patients with large-artery atherosclerosis and small-vessel disease at 3 months. Although the precise causes remain unknown, the initial decreases in EPC numbers after a cerebral ischaemic injury may be attributed to a reduced production, impaired mobilisation or diminished survivability [38-41].

Due to controversies regarding EPC counts, functional assays focusing on the migratory, proliferative, tubulogenic and clonogenic capacity of EPCs (alone or together with EPC counts) may serve as better biomarkers for the prognosis of stroke. Indeed, strong associations observed

between the impaired/diminished EPC function and the extent of infarct volume or post-stroke neurological recovery support this statement and indicate that unless translated into an elevation in functional capacity, increases in EPC number alone may not mean much in terms of functional or neurological recovery. In concordance with this hypothesis, increased function, but not number, of KDR+CD34+CD133+CD45+ cells led to higher endothelial differentiation and better vasculogenesis [42, 43].

Similar to ischaemic stroke, variations in circulating number of EPCs are also reported in patients with SAH and those with cerebral aneurysm or arteriovenous malformations, two main causes of ICH [33, 44-46]. A recent study designed to investigate the influence of CD34+ EPCs on the outcome of ICH has shown that circulating levels of CD34+ cells at day 7 positively correlate with good functional outcome and negatively correlate with residual cavity volume at 3 months [33]. Positive correlations were also observed between CD34+ cell numbers at day 7 and the serum levels of vascular endothelial growth factor (VEGF), angiopoietin-1, brain-derived neurotrophic factor and stromal cell-derived factor-1α (SDF-1α), key factors that affect EPC number and function [33]. These findings suggest that CD34+ progenitor cells may participate in the functional recovery of ICH patients and serve as prognostic biomarkers for ICH and possibly haemorrhagic stroke in general.

It is important to remember that the comorbidities in stroke patients i.e. hypertension, diabetes, coronary artery disease and old age can markedly suppress the number and functional properties of EPCs [47-49]. By compromising the process of cell maturation, the comorbidities may also significantly increase the number of immature EPCs in the circulation and contribute to the pathogenesis of various occlusive arterial diseases [50].

5. EPCs as therapeutics

Failure of clinical trials to replicate positive results obtained in pre-clinical settings with a large number of compounds propose cell-based approaches, capable of responding to a temporally and

spatially changing environment after an ischaemic attack, as valid therapeutic alternatives for ischaemic stroke. Amongst all the cell-based approaches, EPC-based therapies attract a particular attention due to unique ability of these cells to detect and repair endothelial damage and to differentiate into few other cell lines (oligopotency) to promote post-stroke neurogenesis, angiogenesis and vasculogenesis. Despite these, several issues such as effective therapeutic dose and optimal delivery route are likely to affect the efficacy of EPCs in clinical practice [51].

Administration of cells to the same individual who donated the cells (autologous therapy) or to an unrelated donor (allogeneic therapy) represents another important issue in EPC therapy. Naturally, treatments with autologous cells are thought to carry significantly diminished risk of immunological reactions, biological incompatibility and disease transmission. Indeed, intravenous injection of autologous EPCs has been shown to be safe and effective in a randomised, placebo-controlled phase I/IIa trial performed with 18 patients with acute cerebral infarct affecting the middle cerebral artery territory. Compared to placebo-controlled group who received saline or autologous bone marrow stromal cells, EPC group developed fewer serious adverse events and there were no toxicity or allergic reactions in any treatment group during the 4 year follow-up period [52].

Intriguingly, in most clinical studies probing the safety and efficacy of autologous stem cells in ischaemic stroke, bone marrow-derived or adipose tissue-derived mesenchymal cells have been used. While the remaining few studies focused on intracerebral or intra-arterial application of peripheral blood-derived or umbilical cord blood-derived haematopoietic stem cells, only 6 studies registered at the ClinicalTrials.gov (Table 1) have examined or continue to examine the therapeutic capacity or diagnostic and prognostic value of EPCs in ischaemic stroke patients [53, 54].

Other phase I studies conducted with autologous bone marrow derived stem/progenitor cells (BMSCs) or MSCs have also proven the safety of these particular cells in patients with acute and subacute ischaemic stroke and reported no treatment-dependent tumorigenesis, thromboembolism and neurological deterioration during the follow-up period leading up to 5 years [60, 61].

Moreover, intra-arterial infusion of autologous BMSCs to patients between days 5 and 9 of ischaemic stroke has been shown to improve their functional outcome as assessed by Barthel index. In a relevant study, intravenous infusion of autologous EPCs to MCAo rabbits also led to significant improvements in functional outcome and concomitant reductions in infarct size which positively correlated with a decline in apoptotic cell numbers and an increase in microvessel density in the area of ischemic boundary [63]. Contrary to these, despite confirming the safety of BMSCs administered between days 7 and 30, a randomised multicentre study (InveST study) failed to demonstrate any benefit on patients' functional outcome at day 180 [62].

Allogeneic cell therapy, involving transplantation of *ex vivo* expanded cells to an unrelated recipient, addresses the prompt and adequate availability of therapeutic cells. Although the risk of a possible immune reaction with an allogeneic approach may be concerning, discovery of the limited replicative potential of allogeneic cells in the host has somewhat dispelled these concerns [55, 56]. Revelation of equally safe and effective improvement of left ventricular ejection fraction in large animals with ischaemic heart disease by allogeneic and autologous approaches further strengthen the applicability of allogeneic cells in clinical settings, bearing in mind that the larger animal models often more closely mimic human neurological disorders and are therefore considered as a crucial stepping stone for effective therapeutic translation [55, 56, 64]. The findings of MultiStem trial showing greater tissue repair in acute ischaemic stroke patients at one year of receiving intravenously injected allogeneic cells further substantiate this notion and confirm the safety of this therapeutic approach [57]. Interestingly, adjunctive therapy with cyclosporine A to suppress immune reactions attenuated post-stroke cortical injury by promoting the activity and migration of resident neural stem cells and thus accentuated the beneficial effect of EPC-based therapy for stroke [58]. In contrast, immunosuppression by methylprednisolone or cyclosporine appeared to negatively affect the therapeutic role of allogeneic MSCs in treatment of spinal cord injury [59]. Although currently unknown, differences in cell types employed (EPCs vs MSCs) and in diseased tissue (brain vs spinal cord) may partly account for this dichotomy.

Decreases in apoptosis and oxidative stress that markedly influence neuronal viability after an ischaemic injury may somewhat account for the therapeutic effectiveness of EPCs against neuronal degeneration. Indeed, diminished activity and expression of caspase-3 and Bax, a pro-apoptotic enzyme and a pro-apoptotic protein, respectively as well as inhibition of NF-κB, a transcription factor that regulates mechanisms affecting cell survival, cytokine production and oxidative stress, appear to modulate the EPC-mediated neuroprotection in MCAo rats [65]. Recovery of neurotransmitter activity, vascularisation of cerebral tissue and increases in expression and activity of Bcl-2 (an anti-apoptotic protein), superoxide dismutase and glutathione peroxidase (antioxidant enzymes) and glutathione (a non-enzymatic free radical scavenger) also contribute to EPC-mediated attenuation of stroke-evoked cerebrovascular damage [65].

6. Mechanisms responsible for regulating EPC number and behaviour

A better understanding of molecular mechanisms regulating the release, availability, function and interaction of EPCs under physiological and ischaemic conditions is of paramount importance for the design, execution and management of an effective medical therapy aiming to deliver the infused cells to the affected organs in sufficient quantity and for sufficient period of time. Under physiological conditions, a small number of EPCs are constantly released into circulation by bone marrow. However, induction of a localised vascular or ischaemic injury triggers greater mobilisation and release of EPCs into peripheral blood through a complex mechanism involving the excessive release of VEGF. The discovery that EPC and VEGF serum levels peak in patients at day 7 after myocardial infarction and serum level of VEGF at 72 h may predict the increases in EPC level during the first week of ischaemic stroke substantiate the strong correlation between VEGF and EPC levels in ischaemic vascular disease [66, 67]. Progressive increases in serum VEGF and SDF-1α, a chemokine protein, levels were also observed in patients with ICH [33, 68]. As the binding of SDF-1α to CXC chemokine receptor 4 (CXCR4) on EPCs increases recruitment and adherence of EPCs to the ischaemic tissue, therapeutic strategies boosting the availability of

SDF-1α or CXCR4 has been of considerable benefit to potentiate vasculo-reparative role of EPCs in a mouse model of hind limb ischaemia and in db/db diabetic mice [69, 70]. Accumulating recent evidence implicate PI3K, serine/threonine kinase Akt and nitric oxide (NO) in SDF-1α/CXCR4 axis-mediated EPC activation and ensuing improvements in neuro-vascular integrity and function [70, 71].

Oestrogen also intimately affects the EPC characteristics. Re-endothelialisation of carotid artery in ovariectomised mice injected with oestrogen supports the notion that this primary female sex hormone can effectively mobilise and direct EPCs to the site of vascular injury and provides further evidence as to why premenopausal women are better protected against vascular disease compared to similar age men and postmenopausal women. However, inability of oestrogen to restore endothelial integrity in eNOS-deficient mice proves endothelial NO synthase (eNOS) and its end-product NO as important prerequisites for the adequate release or proper function of EPCs [72, 73].

Mobilisation and homing of sufficient numbers of injected EPCs to the site of vascular injury are also influenced by both physical factors (vascular structure, vascular density and flow rate) and differential expression of cell surface adhesion molecules, in particular intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), where chemotactic agents may help determine the final location of infused cells. Increased availability of cytokines, in particular interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) also regulate EPC counts and behaviour after an acute ischaemic injury. For instance while specific binding of IL-6 to its receptor IL-6R (*aka* glycoprotein gp80) and then to a gp130 transducing chain enhances adhesion, proliferation, migration and homing of EPCs after an acute cerebral ischaemia, GM-CSF-mediated increases in EPC accounts for the enhanced peripheral and myocardial neovascularisation as observed in rabbits with hind limb ischaemia and in rats with myocardial infarct, respectively [74-77].

Deficiencies in oxygen, glucose and trophic factor availability alongside a concomitant increase in release of reactive oxygen species during the acute phase of a cerebral ischaemic attack would undoubtedly adversely affect the survival and function of EPCs [89, 90] where regulation of anti- and pro-apoptotic protein expressions and growth factor activity (especially those of VEGF, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor) may profoundly negate these deleterious effects [91, 92]. Preconditioning with hypoxia may also potentiate EPC survival and function in ischaemic settings as evidenced by markedly enhanced survivability and neuro-angiogenic activity of hypoxia-exposed BMSCs in a rodent model of ischaemic stroke [93].

The presence of apoptotic bodies at the site of vascular injury is also known to influence recruitment and reparative capacity of EPCs in a positive manner. Lodgement of substantially higher number of EPCs to the retinal lesions with apoptotic bodies corroborate these findings [78]. Considering that apoptotic cells significantly upregulate the expression of a wide range of cytokines (e.g. VEGF, IL-8, IL-6 and TNF-α) and adhesion molecules (e.g. ICAM, VCAM and E-selectin) on endothelial cells, it is reasonable to state that these proangiogenic cytokines, chemokines and adhesion molecules play a critical role in attracting EPCs to the damaged endothelium [78].

7. Mode of application of EPCs

As the incidence of adverse effects and the quantity of cells delivered to the damaged brain regions may be greatly influenced by the mode of application, the optimal route to administer EPCs into recipients remains an important issue. Albeit technically possible to inject cells via intra-arterial, intranasal or intracerebral (stereotactic implantation) routes, intravenous route would be much preferable in clinical practice due to its less invasive nature and ease of use. However, the number of cells homing into the site of injury and partaking in the cerebrovascular repair process with this route may be particularly low owing to entrapment of most injected cells by organs (e.g. spleen and lungs) that filter the blood. Even so, manipulation of adhesion molecules on plasma membrane

may successfully direct and bind the remaining EPCs to the tissue of interest where concurrent application of agents, such as mannitol, that transiently increase BBB permeability to facilitate crossing into brain parenchyma may be of further benefit to increase EPC numbers in the affected neurovascular regions [79, 80]. Similarly, sorting of cells expressing specific surface antigens by FACS may improve homing of cells to the desired locations, for instance, neural stem cells sorted for surface integrin CD49d has been shown to readily detect and bind to the area of stroke and improve behavioural recovery in mice [81].

Despite bypassing pulmonary circulation and thus ensuring that a substantial number of EPCs reach ischaemic brain to potentiate angiogenesis, intra-arterial delivery is inherently associated with an elevated risk of arterial occlusion, micro-embolism and stroke. Besides, observation of similar rates of functional recovery prompted by intravenous and intra-arterial delivery of autologous bone marrow mononuclear cells after acute ischemic stroke cast a doubt on the selection of arterial route [82, 83].

Intracerebral injection or stereotactic implantation of EPCs directly to the site of cerebral injury may be the most effective method to attenuate neurovascular damage. A randomised, single blind controlled study performed with autologous CD34+ EPCs ($3\text{-}8x10^6$ cells) in a cohort of 30 patients with middle cerebral artery infarction has proven the feasibility of this route. Absence of severe adverse events alongside the significant improvements determined in neurological and functional outcomes by European Stroke Scale, National Institute of Health Stroke Scale (NIHSS) and modified Rankin Scale (mRS) at the end of the 12-month follow-up period prove the safety of this route [84]. Nonetheless, issues pertaining to repeatability and invasiveness make the routine use of this route rather difficult.

A non-invasive but brain-specific delivery of EPCs by intranasal route represents another therapeutic possibility. Mesenchymal Stem Cells (MSCs) or nerve growth factor administered through this route has been shown to improve post-stroke neurovascular regeneration, angiogenesis and functional recovery in mice and rodents [85-87]. Intranasal delivery is a simple

method that can be readily repeated and has the advantage of bypassing the BBB entirely. However, bearing in mind the anatomical differences between animal and human nasal epithelia, further research addressing the specific concerns, regarding the time frame, the behaviour of EPC at the site of delivery, the migratory pathways involved (olfactory nerve vs trigeminal nerve) and the overall efficacy of this route, is required before moving to clinical settings [88].

8. Current concerns associated with EPC treatment

As indicated above, mobilisation and homing of injected EPCs to the site of vascular injury in sufficient numbers constitute two of the main concerns associated with EPC-based treatments. Successful tracking of cells *in vivo* also remain an unresolved critical issue. Although many agents such as iron containing agents, magnetodendrimers, fluorescent proteins and paramagnetic particles (e.g. Gd-DTPA) can be used to track cells *in vivo* by MRI, various advantages and disadvantages pertaining to labelling rates, toxicity, sensitivity and production are documented with each agent. For instance, magnetodendrimers require specialised technical skills to manufacture, paramagnetic substances adversely affect cellular functionality while labelling methods rely on endocytosis or lipofectamine yield notoriously low-labelling rates [65, 94-96]. Indeed, the application of microbeads and quantum-dot-based nanoparticles appears to be superior to flow cytometry in assessing the active changes in the quantitative and functional aspects of EPCs under ischaemic or inflammatory conditions through analysis of microvesicles secreted from EPCs. Furthermore, Dex-DOTA-$Gd3^{+}$ and ^{111}In-oxine radioactive markers have been used in other studies to monitor the fate of transplanted EPCs or to examine their survival period in different models of ischaemic injury including rat cerebral and hind limb ischemia [35, 97, 98].

Although many clinical studies demonstrate the safety and feasibility of stem cell therapy for stroke and report the absence of an association between cell-based treatments and the development of acute infusional toxicity, organ system complications, mortality, infection or malignancy, the possibility of elevated rates of mortality or cerebral haemorrhage due to maladaptive angiogenesis

triggered by comorbidities should always be taken into consideration throughout the therapy [35, 47, 48, 99].

9. Outgrowth Endothelial Cells

Cell culture, based on adhesion of cells to specific substrates, namely fibrinogen or collagen, prior to culture in endothelial cell specific media supplemented with foetal bovine serum and all the necessary growth factors (fibroblast growth factor, VEGF, insulin-like growth factor, etc), hormones (hydrocortisone), ascorbic acid and heparin is considered as the best methodology to obtain a large number of homogeneous EPCs [100]. This *in vitro* approach generates two morphologically and functionally distinct EPC subtypes, early EPCs (eEPCs) and outgrowth endothelial cells (OECs). Early EPCs possess spindle-shaped morphology, appear first in cultures and do not exhibit typical endothelial characteristics e.g. do not form adherens junctions [101]. Even so, current data suggest that eEPCs may contribute to process of endothelialisation and vasculogenesis by secreting several vasoactive substances including cytokines, NO and SDF-1 [102, 103]. In contrast, OECs appear later in culture (14-28 days after seeding) and as shown in figure 2 display typical endothelial cell appearance i.e. cobblestone morphology, form palisading colonies and possess tubulogenic capacity [101, 104]. They also possess higher proliferative and migrational capacities and continue to express progenitor cell markers, CD34 and CD133 which collectively indicate that OECs constitute a separate cell line and are not circulating endothelial cells that simply shed from the vascular wall [101, 105].

10. OECs as therapeutics

As indicated above, EPCs have the physiological features to preserve vascular homeostasis in post-ischaemic settings through neovascularisation and repair of the injured endothelium. However, the limited availability of true EPCs in peripheral blood compromises their therapeutic capacity and necessitates *ex vivo* expansion to produce large quantities of homogenous cells, i.e. OECs that can

be used immediately or cryopreserved for future use. Since, OECs display limited replicative potential *in vivo*, they are recognised as safe therapeutic components [106] and since they are able to detect and home into host endothelium *in vivo*, they are also recognised as efficacious therapeutic components. Indeed, human OECs administered into a murine model of retinal ischaemia by intra-vitreous injection have been shown to incorporate into the resident vasculature within 72 h of injection where they increase normal retinal vasculature, decrease avascular areas and suppress pathological pre-retinal neovascularisation [105]. Again, through direct incorporation into the host endothelium, OECs have been shown to significantly improve cardiac function in a porcine model of acute myocardial infarction [107]. Similarly, OECs injected into the systemic circulation of NOD/SCID mice have been shown to lodge and survive in nine different vascular beds such as gut, heart, liver, lung, spleen and bone marrow for up to 7 months with no ill effects such as thrombosis or infarcts [108]. Taken together, these findings imply that, in addition to being safe and feasible, treatments with OECs are also instrumental in evoking functional benefits [105-109]. Indeed, through direct participation in re-endothelialisation process, OECs attenuate endothelial dysfunction and promote cerebral angiogenesis and vasculogenesis which collectively diminish infarct volume and neurological deficits in settings of acute ischaemic stroke [104, 109]. Suppression of inflammatory responses and the regulation of migratory and proliferative features of vascular smooth muscle cells to mitigate neointimal hyperplasia may represent other pathways involved in the neuro-vascular benefits realised by OEC-based treatment regimens [110-112].

The reparative function of OECs expanded for therapeutic purposes are likely to be affected by the physio-pathological status of the individuals who donate them. It is reasonable to assume that OECs obtained from younger and healthy donors may display better functional capacity compared to those obtained from elderly donors or patients with acute or chronic illnesses. Contrary to this assumption, the hitherto collected data of our ongoing clinical trial demonstrate that EPCs obtained from the peripheral blood of elderly patients with lacunar or cortical stroke establish higher number

of colonies and functional OECs than those obtained from young (18-64 years of age) or elderly (≥65 years old) healthy volunteers [113]. Greater attenuation of cerebral ischaemic damage in experimental models treated with BMSCs obtained from stroke rats supports the notion that previous exposure to ischaemia profoundly augment the neurovascular restorative capacity of OECs [114, 115]. In concordance with these findings, hypoxia-exposed cells produced better angiogenic responses in rats subjected to unilateral hind limb ischaemia. Similarly, exposure of healthy volunteer EPCs to hypoxia in *in vitro* conditions promote their differentiation and as a consequence improve their vasculogenic capacity [29, 116]. In this context, manifestation of higher tubulogenic capacity by OECs obtained specifically from subacute stroke patients denotes that both EPC characteristics and the characteristics of the intrinsic factors that modulate them may oscillate in a time-dependent fashion after an ischaemic event [29]. By repeated sampling from the same patients enrolled for the study, we are currently investigating whether EPC levels and functional aspects correlate with the bioavailability of major growth factors (e.g. VEGF and PDGF-BB), inflammatory cytokines or chemokines (e.g. TNF-α, GM-CSF and IL-12), anti-angiogenic elements (endostatin, angiostatin and thrombospondin-1) and total anti-oxidant capacity during acute, subacute and chronic phases of stroke [113]. Present data imply that transplantation of OECs during the subacute phase of stroke may be the most effective therapeutic approach and a shift in ischaemic status may dramatically accentuate or attenuate their function.

The quantity of blood sample, the presence of comorbidities and the nature of routinely taken medicines invariably influence the success of OEC isolation and the number of OECs obtained. For instance, while diabetics are bound to have fewer number of EPCs, those individuals taking statins will have considerably higher EPC numbers [117-119]. Furthermore, the tissue or haematological source of OECs may also greatly influence their function in that OECs originate from cord blood display longer life spans and shorter doubling times in culture and therefore reveal higher proliferative potential than those originate from adult peripheral blood [120]. Moreover, they also display greater capacity to form vascular networks and are safely stored in cryobanks

with no alterations to their growth potential, phenotype, and karyotype. Nevertheless, *ex vivo* expansion of cord blood-derived outgrowth endothelial progenitor cells on clinical scale elicits high incidence of karyotype aberrations [121].

In addition to being efficacious therapeutics in their own right, OECs may also act as important carriers for gene therapy. The tissue in which the cells settle may dramatically impact the efficacy of both OEC alone-based approaches and gene therapies. This is because some vascular beds provide better microenvironments for the long-term maintenance and proliferation of OECs to which various structural and physiological factors such as shear stress, flow rate and the varied availability of cell-surface adhesion molecules and the chemotactic agents are likely to contribute. Even so, only few studies have probed whether progenitor cells specifically target the organs to home to or simply land and proliferate in microenvironments that are conducive for this. Investigation of the homing pattern of human OECs in NOD/SCID mice has shown that despite lodging mostly in mouse lungs at 3 h, the distribution of cells by 24 h was similar across 9 organs studied, including the heart, liver, lung and spleen where OECs continued to expand up to 7 months without causing any noticeable side effects such as organ toxicity [108]. It is of note that pre-treatment of mice with specific antibodies for E-selectin, P-selectin or anti-α4 integrin prior to OEC injection significantly reduced the number of OECs in lungs at 3 h and OECs from older cultures expanded equally well *in vivo* as younger OECs [108].

It is expected that therapeutic approaches designed to enhance the survival and engraftment of OECs would substantially augment the efficacy of gene therapy. Observation of significantly reduced infarct size and the increased vascularisation in the damaged myocardial region of animals injected with cardiac progenitor cells overexpressing Pim-1, a cardio-protective kinase capable of enhancing cell survival and proliferation, corroborate this hypothesis [122, 123]. These functional benefits coupled with the greater levels of cellular engraftment lasting up to 32 weeks confirm that enhancement of cellular survival, proliferation and regeneration by *ex vivo* gene delivery can

successfully address some of the limitations associated with cell-based therapeutic approaches [122].

As stem cells derived from separate biological sources play distinct therapeutic roles, it is possible that co-transplantation of OECs with different stem cells may enhance their therapeutic effects in stroke patients [131]. A recent study comparing the neurological impact of neural stem/progenitor cell (NSPC) and MSC co-transplantation in 8 patients with ischemic stroke has shown that both patients receiving either 4 intravenous injections of MSCs ($0.5x10^6$/kg body weight) or one intravenous injection of MSCs $0.5x10^6$/kg body weight followed by three injections of MSCs at $5x10^6$/patient and NSPCs at $6x10^6$/patient through the cerebellomedullary cistern had significant improvements in neurological functions and daily living abilities with no sign of tumourigenesis during the 2-year follow-up period. Albeit important in demonstrating NSPC and MSC co-transplantation as an important therapeutic option to improve neurological outcome, further studies with larger sample size, longer follow-up periods and control groups are required to confirm these findings [131]. However, studies reporting elevated survival or reduced tumourigenesis rates achieved by co-transplantation of NSPCs with adipose tissue-derived stem cells and co-transplantation of bone marrow-derived stromal cells with embryonic stem cells already exist [132, 133]. Application of OECs with a non-stem cell substrate may also increase their longevity and restorative capacity. Increased survival of bone marrow-derived stromal cells co-administered with growth factors that provide a scaffold for cell adherence serves as a good example to this type of combinatory approach [134]. Although therapies combining OECs with other stem cells or non-stem cell substrates remain to be carried out, the idea to improve stem cell survival while attenuating an array of adverse events may be a norm in the future.

Given that cell-based therapies are potentially associated with various adverse effects including tumour formation or immunological reactions, cell-based but cell-free strategies may also be considered in regenerative stroke medicine. Indeed, paracrine factors secreted by EPCs/OECs could initiate, modulate and potentiate neuro-angiogenesis after an ischemic insult [135]. An

experimental study investigating this particular question in a mouse model of permanent focal cerebral ischemia has shown that injection of both EPCs or cell-free EPC conditioned media one day after stroke equally enhances peri-infarct capillary density and functional outcome (assessed by forelimb strength test) at 2 weeks after stroke [136]. In accordance with these findings, both bone marrow-derived autologous OECs and conditioned media obtained from their culture inhibited proliferation and migration of vascular smooth muscle cells *in vitro* and regulated their arrangement [110]. Transfusion of OECs soon after an injury into rabbit ear canal artery in this study abated increases in intimal layer by mitigating the early inflammatory and angiogenic responses [110].

11. OEC senescence and functional aspects

Safe and efficient *ex vivo* expansion of EPCs from single figures to hundreds of millions is a lengthy process requiring several weeks and an important prerequisite for their therapeutic application. Shortly after their *in vivo* administration, OECs, due to their limited replicative potential, go into senescence and display the typical signs of ageing i.e. telomere shortening and low telomerase activity [106]. Albeit useful in minimising the risk of tumourigenesis, OEC senescence is also associated with functional impairment. Hence, effective modulation of mechanisms involved in this process can both extend OECs' therapeutic window and accentuate their reparative potential. Indeed, silencing one of the proven modulators of OEC senescence, namely IL-8 by shRNA delayed OEC ageing and as a result enhanced their proliferative and vasculoreparative capacity [106]. In agreement with this finding, knocking down of the IL-8 receptor beta (IL-8RB) has also alleviated both replicative and oncogene-induced senescence in fibroblasts [124].

A substantial body of evidence indicates that oxidative stress can also induce the ageing process in endothelial cells where an association between oxidative stress, accelerated telomere shortening and senescence is demonstrated through studies manipulating the intracellular redox state via a

vitamin C analogue, homocysteine or glutathione [125-127]. Although NO is thought to counteract endothelial cell senescence by concomitantly stimulating telomerase activity and reducing telomere erosion, experiments using pharmacological tools (e.g. NO donors and NOS inhibitors) or silencing RNA technology to manipulate NO levels showed no effect on telomerase activity, cellular replicative capacity or the accumulation of senescent cells [128-130].

12. Issues to consider while designing future clinical trials

Accumulating data from phase I and II studies suggest EPC-based treatments as efficacious options for ischaemic stroke. Although the reduced risk of rejection may advocate selection of autologous cells for the so-called safer therapy, the limited availability of EPCs in biological niches such as peripheral blood and bone marrow seriously hinders their sufficient isolation and thus rules out their application as autologous therapeutics for acute ischaemic stroke. Considering the apparent lack of HLA-class II antigens on allogeneic cells and therefore the reduced likelihood of antigen-specific immune reactions, EPCs can be safely expanded *ex vivo* and used as allogeneic therapeutics [137, 138]. However, the requirement for a subsequent OEC infusion and patients' tolerance to repeat dosing need to be taken into consideration while contemplating treatments with allogeneic cells and administration of cells from a different donor may be necessary to avoid anamnestic reaction [56]. Larger randomised clinical trials closely monitoring the long-term immunological profile of participants treated with allogeneic OECs would produce the rigorous clinical evidence required for transforming these cells to an off-the-shelf type of therapeutics for the treatment of ischaemic stroke.

It is evident from the literature that the availability of relevant previous preclinical and clinical data, patients' characteristics (type, severity and phase of stroke), clinical question posed (e.g. testing safety vs efficacy of cells), mode of delivery as well as the purity and quantity of isolated or expanded cells will shape the design of future clinical trials. For example, intra-arterial and intravenous administration of cells during early stages (acute to subacute) of stroke is carried out

to attenuate acute tissue injury [110-112, 135] while targeted intracerebral administration of cells during chronic stages of stroke is performed to enhance vasculogenesis and neurogenesis through direct engraftment to the brain tissue [84]. Unlike experimental stroke where lesions consistent in size and location are generated by a reasonably well-controlled occlusion of a cerebral artery, substantial inter-individual variations in size and distribution of stroke lesions exist in humans. These inevitably affect patients' selection for certain trials, particularly those involving stereotactic implantation of cells.

The mode of delivery will also affect the possibility of blinding participants or researchers to specific treatments. For instance, due to potential hazards associated with intra-arterial and intra-cerebral routes, recruitment of healthy volunteers as study controls may not be adequately justified. Instead, serial neuro-radiological assessments covering pre-treatment, intra-treatment and post-treatment periods may be considered to reduce both intra-individual and inter-individual variance. In fact, intracerebral injection during the acute phase of stroke may even be counterproductive considering that changes in neuro-inflammatory events, lesion size and oedema volume continue to occur throughout the earlier phase of the disease. Administration of OECs through an alternative route during acute stroke, on the other hand, would help inhibit the initial steps of the ischaemic cascade, augment neurovascular protection and allow assessment of neurological and functional recovery using a set of valid and reliable clinical scales such as NIHSS, mRS or Barthel Index.

In addition to administration of OECs, future studies should also focus on development of novel agents or approaches that can mobilise and integrate endogenous EPCs into new or pre-existing vessels to help post-stroke cerebral regeneration. Discovery of reliable biomarkers pinpointing the endothelial cell or BBB damage and highlighting the involvement of EPCs in neurovascular repair after stroke would be of great help in the diagnosis and management of stroke. As *in vivo* and *in vitro* hypoxia appear to precondition OECs to the microenvironment of the infarcted tissue (brain), it is also important to test the safety and efficacy of OECs grown under hypoxic conditions in future clinical trials [92, 115, 116].

13. Conclusions

Loss of endothelial integrity and impaired capacity for neovascularisation are thought to contribute to ischaemic events affecting various organs including the brain [22-28]. Accumulating recent studies demonstrate that re-endothelialisation of damaged arteries not only requires the migration, proliferation and sprouting of pre-existing mature endothelial cells but also relies on the bioavailability of functional EPCs which can promote endothelial repair directly by differentiation into endothelial cells or indirectly by regulating secretion of various elements, like VEGF, that individually or collectively augment the reconstructive role of progenitor cells [29].

Despite some issues regarding the precise morphological and functional characteristics of EPCs remain uncertain, rapidly accumulating preclinical and clinical findings indicate that EPCs may serve as therapeutics in treatment of several disorders culminating from perturbed endothelial integrity and function [34, 35]. Indeed, through restoration and maintenance of vascular endothelium by constant replacement of dead or dying endothelial cells and concomitant induction of angiogenesis, neurogenesis and vasculogenesis, EPCs help preserve vascular homeostasis at all times and improve patients' neurological and functional outcome after a brain injury. However, consensus on their definition are needed.

Ex vivo expansion of EPCs leading to generation of a vast amount of homogenous OECs, equipped with significant tubulogenic, migratory, proliferative, neurovasculo-reparative capacity opens a new avenue for the treatment of stroke. Possibility of allogeneic application of OECs backed up by findings demonstrating the absence of immune responses due to lack of HLA-class II antigens on these particular cells is encouraging. It is anticipated that well-thought laboratory and translational studies considering the cell source, mode of action, optimal cell dose, timing of application, comorbidities, cell senescence, tumourigenicity and immunogenicity during the design phase will significantly enhance our understanding of OECs' therapeutic capacity and pave the way for future clinical studies assessing their efficacy beyond the acute phase of stroke.

Conflict of interest

The author declares no conflict of interest.

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Figure Legends

Figure 1. From risk factors to clinical manifestation of stroke.

Uncontrolled modifiable and non-modifiable risk factors play a prominent role in the pathogenesis of both ischaemic and haemorrhagic strokes. Ischaemic strokes are further divided into two subgroups depending on the aetiology of the disease; embolic stroke or thrombotic stroke. Haemorrhagic strokes are also further divided into two subgroups depending on the location of bleeding, intracerebral haemorrhage (ICH) or subarachnoid haemorrhage (SAH). Size and location of the stroke lesions or haemorrhage determine the type and severity of functions regulated by different cerebral lobes in physiological settings.

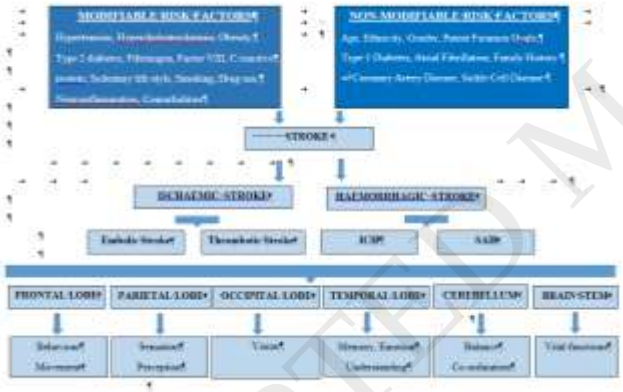

Figure 2. Flow cytometry and functional analyses of EPCs obtained from the peripheral blood of a healthy volunteer.

Mononuclear cells separated from other components of peripheral blood by centrifugation on a density gradient media prior to treatment with an Fc receptor blocker. Cells were then incubated with a fluorescein isothiocyanate-, peridinin-chlorophyll-, allophycocyanin- and phycoeryhtrin-conjugated antibodies to detect CD45 (a marker for haematopoietic cells), CD34, CD133 and KDR antigens. Flow cytometry shows the prevalence of CD34+ (**A**), CD34+CD133+ (**B**) and CD34+CD133+KDR+ (**C**) cells in a population of cells negatively stained for CD45. In additional

experiments, separated mononuclear cells seeded on fibronectin-coated plates and cultured in endothelial growth medium supplemented with all necessary ingredients including a cocktail of growth factors (e.g. fibroblast growth factor, VEGF and insulin-like growth factor), hydrocortisone, heparin and serum. Upon removal of non-adherent cells from the culture after 48 h, cells continued to be cultured in the same medium until endothelial colony-forming cells with true progenitor characteristics appeared (**D**). Similar to mature endothelial cells, outgrowth endothelial cells display cobblestone morphology (**E**) and form tubes on matrigel, a marker of *in vitro* angiogenic activity (**F**).

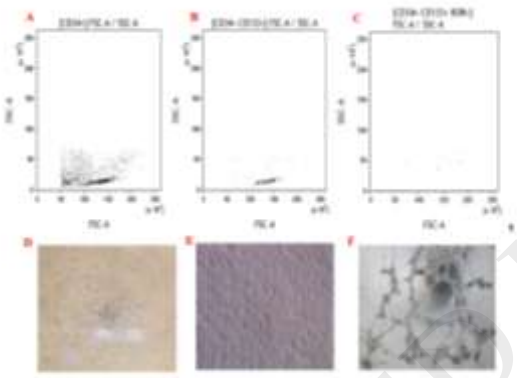

Table 1

Clinical trials with endothelial progenitor cells in ischaemic stroke.

NCT Reference	Study type	Estimated sample size	Start date	Chief Investigator	Study population
NCT03218527	observational	120	10.2015	Pol Camps-Remom	Adult patients presenting at least on atherosclerotic plaque in the internal carotid artery ipsilateral to stroke
NCT02980354	observational	200	02.2017	Ulvi Bayraktutan	Elderly patients (≥65 years of age) with lacunar or cortical stroke. Elderly and young (18-64 years old) healthy volunteers.
NCT02157896	observational	30	05.2013	Hao Chen	Adult patients with infarcts within the territories of middle cerebral artery, posterior cerebral artery, anterior cerebral artery, vertebrobasilar area or watershed area.
NCT01468064	interventional	20	11.2011	Zhenzhou Chen	Patients with acute cerebral infarcts within the middle cerebral arterial territory received autologous BMSCs, autologous EPCs or placebo.
NCT02605707	interventional	30	11.2014	Zhenzhou Chen	Patients with chronic ischaemic stroke received intracerebral transplantation of autologous endothelial progenitor cells
NCT01289795	observational	30	07.2010	Thomas Liman	Adult patients with first-ever acute ischaemic stroke