

REVIEW

Therapeutic revascularisation of ischaemic tissue: the opportunities and challenges for therapy using vascular stem/progenitor cells

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Abstract

Ischaemia-related diseases such as peripheral artery disease and coronary heart disease constitute a major issue in medicine as they affect millions of individuals each year and represent a considerable economic burden to healthcare systems. If the underlying ischaemia is not sufficiently resolved it can lead to tissue damage, with subsequent cell death. Treating such diseases remains difficult and several strategies have been used to stimulate the growth of blood vessels and promote regeneration of ischaemic tissues, such as the use of recombinant proteins and gene therapy. Although these approaches remain promising, they have limitations and results from clinical trials using these methods have had limited success. Recently, there has been growing interest in the therapeutic potential of using a cell-based approach to treat vasodegenerative disorders. In vascular medicine, various stem cells and adult progenitors have been highlighted as having a vasoreparative role in ischaemic tissues. This review will examine the clinical potential of several stem and progenitor cells that may be utilised to regenerate defunct or damaged vasculature and restore blood flow to the ischaemic tissue. In particular, we focus on the therapeutic potential of endothelial progenitor cells as an exciting new option for the treatment of ischaemic diseases.

Background to cell therapy

Ischaemia is characterised by a reduction in oxygen supply to tissues and organs, usually as a result of blood

vessel constriction or obstruction. This leads to hypoxia and tissue damage as a consequence of the build up of waste metabolites and may result in cell death [1]. Many important diseases are characterised by acute or chronic ischaemia, which affect millions of people each year and represent a considerable morbidity, mortality and economic cost to healthcare systems worldwide [2].

The use of cell therapy for vascular regeneration offers an exciting new prospect in regenerative medicine. Indeed, in the field of vascular biology there are already a considerable number of ongoing clinical trials using a cytotherapy for ischaemic diseases such as myocardial ischaemia and peripheral limb ischaemia [3,4]. However, the delivery of the correct cell type to the precise area of injury or vascular insufficiency is difficult and many factors need to be considered.

One such factor to consider is efficacy. Cells for vascular therapy must be able to home to ischaemic or damaged tissue and engage in vessel formation alone or in unison with resident vasculature to achieve a controlled and functional reperfusion event, without causing pathological angiogenesis (for example, proliferative retinopathy in the vitreous of the eye).

The timing of delivery and cell numbers also require consideration. A cell therapy approach should be aimed at promoting revascularisation of ischaemic tissue. There is a therapeutic window in which to deliver the cells, to avoid extensive tissue damage, fibrosis and necrosis. The evaluation of the most suitable timing of cell delivery as well as the number of cells needed to integrate into resident vasculature and promote revascularisation of specific tissues requires careful optimisation and evaluation.

A third factor is the administration route. An important point to consider when examining cell recruitment is the mode of cell delivery. Previous studies using vascular progenitor cells have shown that local delivery results in increased homing as the cells are directly delivered to the ischaemic area or tissue environment that is experiencing the disease [5]. A systemic delivery strategy is based on

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the capacity of the cells to be mobilised and directed via chemokines to the ischaemic area; however, the drawback of this approach is that this may result in cells localising to non-target organs such as the liver, kidneys, spleen and lung.

Finally, one should consider cell choice, a critical aspect of any cell therapy. The correct cell must be chosen for its phenotype, cell characteristics and biological functions. This is important, because some ischaemic diseases have added complicating factors such as a hypoxic and pro-inflammatory microenvironment. In this situation, injecting any cell with the predisposition to switch to an inflammatory phenotype could exacerbate the underlying pathology [6].

Bone marrow (BM) contains a great variety of stem and progenitor cells, such as haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). BM therefore represents a relevant source of vascular progenitor cells. Clinical trials have tested BM-derived unfractionated mononucleated cells as a therapy for various ischaemic disorders such as heart disease [7]; however, results from these studies have generated conflicting results. This is largely due to the fact that BM contains a heterogeneous mix of cells, making the evaluation of the relative contribution of specific cell types very difficult. Two other accessible sources for isolation of stem/progenitor cells are adult peripheral blood and umbilical cord blood.

There are many cell types currently being considered for cytotherapies in the context of ischaemic diseases (Figure 1). Such cells include MSCs [8], multipotent adult progenitor cells (MAPCs) [9], EPCs [10], pluripotent embryonic stem cells (ESCs) [11], and induced pluripotent stem cells (iPSCs) [12] (Table 1).

The differentiation potential of MSCs and MAPCs into mural cells such as smooth muscle cells (SMCs) and pericytes has been reported [13,14]. Mural cells play a key role in the context of vascular regeneration by providing structural support to the vasculature, and regulating new blood vessel formation, maturation and stabilisation. It has been suggested that building a new blood vessel requires the interaction of both endothelial cells and mural cells. The present review will particularly focus on the endothelial cell component of cytotherapies for vascular regeneration. The three main cell candidates are ESCs, iPSCs and EPCs.

Pluripotent stem cells

Pluripotent stem cells have generated widespread attention in the last decade due to their capacity to become virtually any cell in the body. With the exception of extra-embryonic tissue, pluripotent stem cells have the potential to differentiate into derivatives of all three germ layers.

Embryonic stem cells

ESCs are derived from the inner cell mass of a pre-implantation blastocyst-stage embryo, a process that results in the loss of the embryo. This source presents an ethical dilemma for many, but recent improvements have demonstrated that ESCs can be generated from a single blastomere, without damage to the embryo [15]. Beyond the ethics dimension, there are major concerns about the therapeutic use of ESCs due to an inherent risk of tumour or teratoma formation [16].

Analysis of human ESCs differentiation suggests that endothelial cells originate via a primitive haemangioblast precursor that can give rise to cells of both haematopoietic and endothelial fate [17]. hESC-ECs have been differentiated using a variety of methods, including co-cultures with stromal layers [18] or in suspension culture as embryoid bodies [17,19]. These hESC-ECs express a variety of endothelial markers (PECAM1, CD34, KDR and VE-CAD), uptake DiI-Ac-LDL, display the typical cobblestone morphology and form tubes in Matrigel [20]. Transplantation of hESC-ECs has shown that these cells can evoke reperfusion in animal models of hind limb ischaemia [21,22] and ischaemic heart disease, demonstrating their promising therapeutic potential in promoting neovascularisation [23]. However, before these cells can be used as a therapy for patients suffering with ischaemic vascular disease, the generation of immune-compatible transplants needs to be addressed so that clinical application can be realised.

Induced pluripotent stem cells

Even following the transplantation of closely matched graft cells, a patient is still likely to require immunosuppressive therapy. Autologous cell transplantation is therefore highly desirable to overcome immunogenic mismatch between host and graft.

In 1989, Weintraub and colleagues [24] demonstrated that it was possible to drive fibroblast cell fate into muscle, by expressing the transcription factor MyoD. This phenomenon was later exploited by Takahashi and Yamanaka in mouse fibroblasts [25], and later in human cells to induce the 'reprogramming' of adult skin fibroblasts to a pluripotent state using C-MYC, KLF4, OCT3/4 and SOX2 [26]. These cells, known as iPSCs, have many of the advantages of ESCs, and critically can be generated in a patient-specific manner.

It is possible to differentiate endothelial cells from human iPSCs; differentiation protocols that have been established for human ESCs are applicable to iPSCs. These include, culturing iPSCs with OP9 as feeder cells [27], embryoid body assays [28] and exposure to vascular endothelial growth factor [29]. These culture conditions initiate differentiation to an endothelial phenotype with iPSC-ECs displaying typical endothelial characteristics,

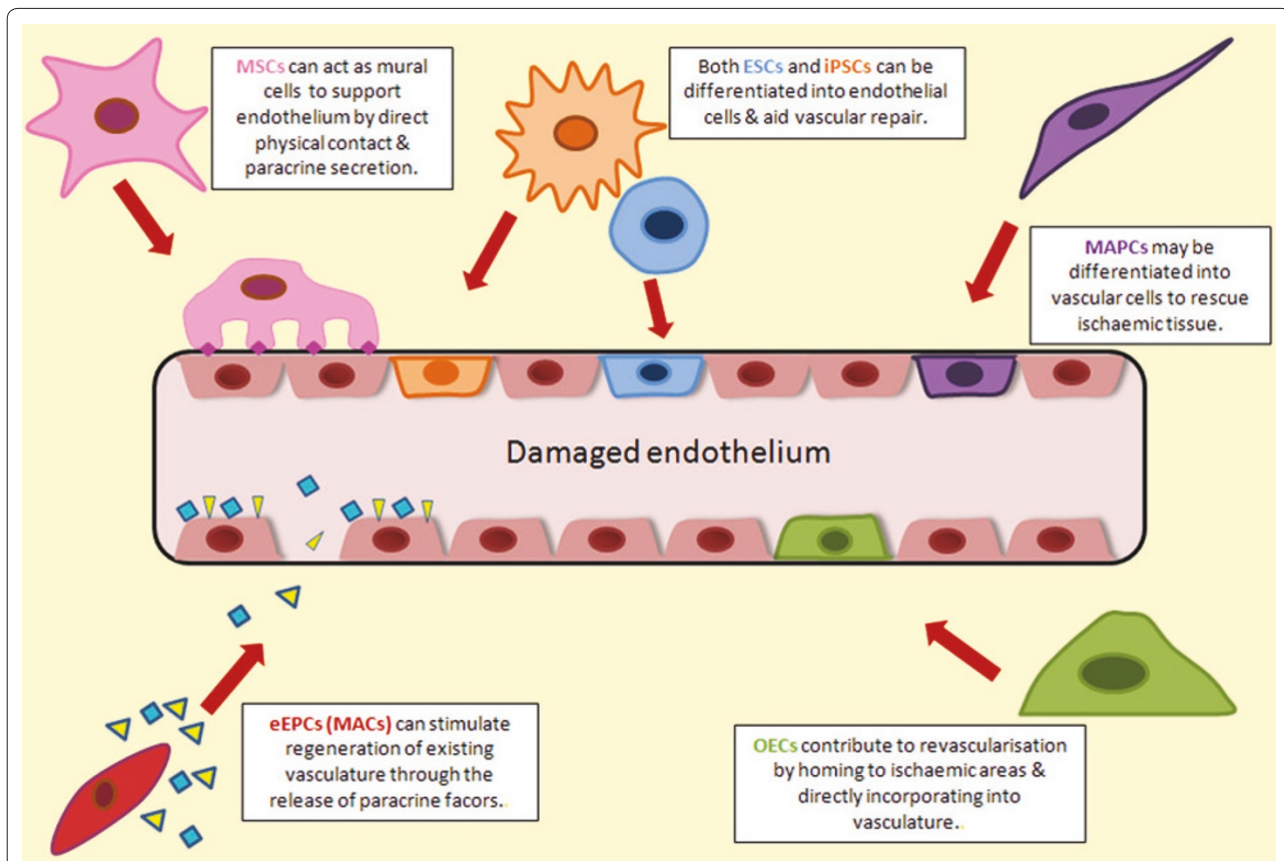


Figure 1. Schematic representing the role of stem and progenitor cells in vascular repair. Multiple stem and progenitor cells may contribute to vascular repair *in vivo*. Both embryonic stem cells (ESCs; blue) and induced pluripotent stem cells (iPSCs; orange) can be differentiated into vascular cells and may be utilised *in vivo* as endothelial cells with the potential to engraft into damaged or ischaemic host vasculature. Mesenchymal stem cells (MSCs; pink) have the potential to differentiate into mural cells such as pericytes and smooth muscle cells. This would be particularly useful in ischaemic tissue as mural cells are essential for stabilisation of newly formed vessels and communicate closely with endothelial cells through adherens junctions. The protein N-cadherin is depicted as pink diamonds. Multipotent adult progenitor cells (MAPCs purple) may also be differentiated into endothelial cells to aid vascular repair and reduce ischaemia. Early endothelial progenitor cells/myeloid angiogenic cells (eEPCs/MACs; red) play a paracrine role by secreting pro-angiogenic growth factors and cytokines (yellow triangles and blue squares) to stimulate vascular regeneration. Outgrowth endothelial cells (OECs; green) display a typical endothelial phenotype and have clinical potential for ischaemic disease as they home to ischaemic areas and directly integrate into denuded endothelium.

Table 1. Characteristics of stem/progenitor cells that can be used for therapeutic revascularisation of ischaemic tissue

Cell type	Proliferative potential	Risk of tumour formation	Capacity to differentiate into vascular cells	Engraftment/angiogenic potential <i>in vivo</i>
Pluripotent stem cells				
Embryonic stem cells	+++	+++	+	+
Induced pluripotent stem cells	+++	+++	+	+
Adult stem cells				
Mesenchymal stem cells	+	+/-	++	++
Multipotent adult progenitor cells	++	+/-	++	++
Endothelial progenitor cells				
Early endothelial progenitor cells	+/-	+	+/-	+
Outgrowth endothelial cells	++	+/-	+++	+++

+, ++, +++, Weak/low, moderate and high potential, respectively; +/-, little or no potential

including capillary formation in Matrigel and expression of endothelial markers such as CD31, KDR, CD144 and endothelial nitric oxide synthase. A recent study has demonstrated the therapeutic potential of iPSCs: when injected into an ischaemic hind limb model, iPSCs were shown to increase capillary density and improve blood perfusion [12].

The past 5 years have seen major advances in the potential translation of iPSCs to the clinic, including improvements in reprogramming efficiencies, alternatives to skin fibroblasts, and integration-free reprogramming transgene expression methods such as adenovirus, minicircle DNA, episomes and synthetic proteins and mRNA [30]. However, several further issues must be resolved before iPSCs can be used clinically. There are concerns with iPSCs regarding the use of the proto-oncogene *c-myc*, and insertion mutagenesis due to the use of retroviral sequences. There are also concerns regarding the tumourigenic potential of differentiated PSCs [31]. Additionally, there are issues surrounding the genetic and epigenetic integrity of the iPSCs and also the true nature of their immunogenic status. For example, recent reports have concluded that human iPSCs carry an increased mutational load in the form of karyotypic abnormalities and the accumulation of somatic protein coding point mutations relative to the parent cell line used to generate them [32,33]. These mutations are presumed to be due to 'reprogramming stresses'. However, in these studies the genomes of clonally derived iPSCs were being compared with reference genomes generated from a polyclonal parent population and therefore, the identification of *bona fide* mutations could have been hampered. Furthermore, it must be noted that iPSCs appear to retain an epigenetic memory of their former cell type [34]. The influence of this on the differentiation capacity of the iPSCs is not yet clear; for example, persistence of epigenetic memory in iPSCs may be limited during sequential passaging and time in culture. However, this epigenetic memory may be advantageous if we consider deriving iPSCs from vascular cells in order to enhance endothelial cell and smooth muscle cell production from iPSCs.

Adult stem/progenitor cells

Lineage-committed multipotent or unipotent progenitor cells may represent a more feasible cell choice for the treatment of vascular disease. The benefits of using adult-derived progenitors are that they allow for autologous therapy and they are lineage restricted, and thus probably are much safer than pluripotent stem cells with fewer ethical concerns. Many defined populations are already being tested for safety and feasibility in clinical trials, so adult progenitor cells are likely to be the first cells translated to the clinical setting.

Endothelial progenitor cells

EPCs have been extensively studied as progenitor cells capable of contributing to neovascularisation. These cells may also have potential as diagnostic/prognostic biomarkers for cardiovascular and cerebrovascular disease. There appears to be an inverse correlation between the number of circulating EPCs and the number of deaths from cardiovascular events [35]; furthermore, they can also be used as tools to study vascular disease [36]. EPCs may be isolated from peripheral blood, BM and umbilical cord blood. Although they represent only a minor population (0.05 cells/ml blood) relative to the cell populations in whole blood, EPCs have been shown to play a major role in therapeutic angiogenesis and vascular repair in various ischaemic tissues; and represent an important candidate for a cell therapy approach [37].

The first putative EPCs were isolated from human peripheral blood in 1997 by Asahara in a seminal paper that appeared to demonstrate postnatal vasculogenesis [38]. This team, lead by Jeffrey Isner, used a combination of cell-sorting approaches, using CD34 as a marker for EPC selection, followed by subsequent plating on fibronectin to isolate EPCs. These cells were found to express endothelial cell-like markers CD31, VEGFR2, Tie-2 and E-selectin after 7 days in culture, indicating their differentiation towards an endothelial phenotype. Importantly, this study also demonstrated functionality; when the pre-labelled EPCs were injected into the ischaemic limbs of nude mice, they appeared to target avascular zones within the diseased tissue and participate in neovascularisation, thus providing evidence of EPCs angiogenic capacity. Since Asahara's discovery, modified versions of this isolation procedure have been used for isolating EPCs [39] and it is now evident that these EPCs are likely to be a heterogeneous mix of endothelial and haematopoietic cells that have been shown to comprise monocytes and macrophages [40].

Over the last decade there have been contradictory reports surrounding the precise nature of EPCs as pre-clinical and clinical investigations evaluating the therapeutic potential of EPCs have yielded inconsistent results [10,41] This is largely due to the fact that EPCs lack a uniform definition and there are no definitive markers used to isolate an EPC [42]. It is now accepted that there are at least two definitive EPC subsets that can be isolated *in vitro*. One cell type appears after a few days in culture and these cells are called early EPCs (eEPCs), while the other type of cells, appearing much later, are called outgrowth endothelial cells (OECs) or endothelial colony-forming cells (ECFCs) [37]. The biological properties of these two cells, their potential role in vascular repair and their potential for cytotherapy will be further discussed.

Early endothelial progenitor cells

The early EPC population is now generally well recognised not to be endothelial in origin [43]. Although this subset of circulating cells express typical endothelial markers such as CD31, VEGFR2 and Tie2, these markers are not necessarily endothelial specific, and mononuclear cells in culture may acquire these markers through platelet microparticle uptake [44]. Furthermore, eEPCs also express haematopoietic markers CD14 and CD45 and morphologically appear in culture as spindle-shaped cells, with a low proliferative potential, and bear no resemblance to the cobblestone appearance of endothelial cells. Recent research including work from our own laboratory, has demonstrated that early EPCs are distinct from endothelial cells, in terms of their gene expression, proteomic profile and ultrastructure [43]. We have shown that eEPCs represent haematopoietic cells with a molecular fingerprint that resembles pro-angiogenic M2 macrophages [45]. Given all this evidence we feel that the term eEPC is no longer accurate to describe this population of cells, and we recently coined the term myeloid angiogenic cells (MACs) as a more fitting description of the true identity of these cells.

Despite their haematopoietic origin, MACs do have therapeutic value; they appear to stimulate angiogenesis in a paracrine manner [46]. MACs home to areas of ischaemia and stimulate regeneration of existing vasculature through the secretion of pro-angiogenic cytokines and growth factors such as IL-8, hepatocyte growth factor, insulin-like growth factor and granulocyte colony-stimulating factor [45,47]. While these cells do not incorporate directly into the vasculature, they remain proximal or loosely attached to the damaged tissue in a perivascular position. In this manner, MACs have been shown to facilitate vascular repair and promote reperfusion in critical limb ischaemia, ischaemic retinopathy and after myocardial infarction [45,48,49]. However, it must be noted that because these cells are linked to macrophages they may be highly plastic, and therefore in the presence of an inflammatory or hypoxic tissue environment they may enhance inflammation [6]. Therefore, careful consideration of the source of the cells and the milieu must therefore be taken into account if these cells are to be used clinically. It may be more advantageous to fully characterise mechanisms responsible for MACs paracrine effects so that respective approaches can be established to promote vascular regeneration. This will include use of conditioned medium, use of recombinant proteins and stimulation of pro-angiogenic pathways.

Outgrowth endothelial progenitor cells

OECs, also known as ECFCs or late EPCs, represent *bona fide* endothelial progenitor cells [50]. A classic method for obtaining OECs is *in vitro* culture of the mononuclear

fraction of blood at high density on collagen. Using this method, OEC colonies start appearing from 3 to 5 weeks and bear a typical cobblestone-shaped morphology. Research from our own group using genome-wide transcriptomics, proteomics and ultrastructural evaluation has demonstrated OECs intrinsic endothelial identity. OECs have a remarkably high proliferative capacity in comparison with circulating endothelial cells and maintain an endothelial phenotype with *ex vivo* long-term expansion [43].

Functionally, OECs display endothelial characteristics. Previous studies have highlighted the *in vitro* angiogenic potential of OECs; they are capable of integrating into pre-existing vessels and of *de novo* tube formation in several *in vitro* models [51,52]. However, a rigorous test for true endothelial potential is direct engraftment *in vivo*. Various groups, including our own, have demonstrated that OECs possess *in vivo* potential by directly aiding vascular repair and forming well-perfused vasculature in various *in vivo* models [51,53]. Recently, we demonstrated OECs therapeutic potential for retinal ischaemia when they were delivered intravitreally into a mouse model and homed specifically to ischaemic areas within the central retina and integrated directly within the host vasculature, assisting in vascular remodelling by forming vascular tubes [54]. Importantly, this study demonstrated functional benefits such as a significant decrease in ischaemia and a concomitant increase in normal vasculature. Although this study examined the therapeutic benefit of OECs over a relatively short time period of 72 hours, the long-term effectiveness and safety of OECs has also been assessed in a porcine model of acute myocardial infarction [55]. Furthermore, OECs injected into the systemic circulation of non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice are able to lodge and survive in nine different vascular beds for up to 7 months after intravenous tail vein injection, without inducing thrombosis or infarcts [56]. This finding highlights the potential benefits of a novel cytotherapy using a well-defined population of OECs for patients with ischaemic-related pathology.

However, it must be noted that OECs have some limitations when compared with ESCs/iPSCs. Firstly, OECs lack a unique surface marker to identify them, and this limits their isolation using a cell-sorting approach. A panel of surface markers are therefore needed to characterise OECs for expression of endothelial markers such as CD146, VE-cadherin, CD31 and VEGFR2. They also remain a relatively difficult cell to isolate using *in vitro* culture methodologies. OEC colonies can take quite a long time to emerge, with some colonies taking up to 1 month to appear in culture. Once isolated, however, OEC colonies can be expanded to yield a pure population of

cells with a high proliferation rate; for example, some cord blood-derived colonies have been expanded up to a level of 80 population doublings in just 90 days.

Conclusion

There is considerable therapeutic potential in using a cell-based approach to treat vasodegenerative disorders [57]. Indeed, various stem cells and adult progenitors have been highlighted as having important vasoreparative and regenerative potential in vascular medicine [58]. Some of these progenitor cells are already being used in clinical trials for the treatment of ischaemic diseases such as limb and cardiac ischaemia and are showing promising results [4,59]. This review has examined the clinical potential of various cell-based therapeutic approaches that may be applied to regenerate defunct or damaged vasculature and restore blood flow. There are many options of which cell types to use, although ultimately the best options will need to be tailored for disease type, for the precise nature of repair or vessel regrowth being sought and for whether the therapeutic regime is autologous or allogeneic. Ideally, once delivered, these cells should have an unambiguous fate with precise reparative and vessel formation properties *in vivo* whilst having limited replicative potential, thereby reducing the neoplastic risk associated with many stem cell therapies. With a focused research effort in the coming years, there is every expectation that cell therapy can become an important and highly beneficial treatment option for ischaemic disease.

Abbreviations

BM, bone marrow; ECFC, endothelial colony forming cell; EPC, endothelial progenitor cell; ESC, embryonic stem cell; HSC, hematopoietic stem cell; IL, interleukin; iPSC, induced pluripotent stem cell; MAC, myeloid angiogenic cell; MAPC, multipotent angiogenic progenitor cell; MSC, mesenchymal stem cell; OEC, outgrowth endothelial cell; SMC, smooth muscle cell.

Competing interests

The authors declare that they have no competing interests.

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