Endothelial Progenitor Cells' Classification and Application in Neurological Diseases

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Abstract The therapeutic effects of endothelial progenitor cells (EPCs) on ischemic stroke have been extensively studied in recent years. However, the differences in early EPCs and endothelial outgrowth cells (EOCs) are still unclear. Clarifications of their respective properties and specific functioning characteristics contribute to better applications of EPCs in ischemic diseases. In this review, we discuss cellular origin, isolation, culture, surface markers of early EPCs and EOCs and relevant applications in neurological diseases. We conclude that EOCs possess all characteristics of true endothelial progenitors and have potent advantages in EPC-based therapies for ischemic diseases. A number of preclinical and clinical applications of EPCs in neurological diseases are under study. More studies are needed to determine the specific characteristics of EPCs and the relevant mechanisms of EPCs for neurological diseases.

Keywords Early endothelial progenitor cells · Endothelial outgrowth cells · Cell origin · Surface antigen expression · Neurological disease

1 Introduction

Endothelial progenitor cells (EPCs) are circulating cells that display cell surface antigens similar to endothelial cells *in vitro*; these cells circulate and accumulate in areas of ischemia or vascular injury, facilitate the repair of damaged blood vessels, and augment angiogenesis as needed in various tissues [1]. Recently, EPCs have been shown to play a major role in angiogenesis in multiple ischemic

diseases and have therefore emerged as important candidates for cell therapy. There are at least two different populations of EPCs: early EPCs appear 3–5 days after the onset of culture, whereas endothelial outgrowth cells (EOCs) appear 2–3 weeks later [2]. They promote angiogenesis through different mechanisms respectively. Clarifications of their properties and specific functional characteristics contribute to better applications of EPCs in ischemic diseases.

In this review, we discuss the cellular origin, culture, isolation, surface markers, and clinical applications of EPCs and EOCs.

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2 Origin of early EPCs and EOCs

EPCs are mainly located in bone marrow, cord blood, peripheral blood, and vessels. Recently, EPCs have also been shown to be localized in vessels, where they express the specific surface marker CD177 [3]. Additional studies also demonstrated that hematopoietic stem cells (HSCs),

mononuclear cells, mesenchymal cells, myeloid progenitor cells [4], side population cells [5] and embryonic stem cells could also directly differentiate into endothelial cells (ECs), which enriched EPCs sources.

Cord-blood derived EPCs (CB-EPCs) have significantly advantages over EPC derived from other sources. For example, CB-EPCs exhibit longer telomeres, which are associated with high levels of telomerase activity and high proliferation potential. Additionally, these cells express abundant amounts of genes involved in the cell cycle and blood vessel development [6]. CB-EPCs are also relatively tolerant, and transplants using CB induce less severe graft-versus-host disease [7]. Importantly, CB remains the largest source of stem cells available; to date, more than 450,000 unrelated CB units are banked. CB also contains a significantly higher frequency of EPCs than other sources. Finally, CB-EPCs provide the additional benefits of immediate availability of cells, no risk to the donor (noninvasive collection procedures), and low risk of infectious disease transmission [8].

3 Surface markers, isolation, and identification of early EPCs and EOCs

The typical surface marker expressed by early EPCs is CD133 [9]. Ormiston et al. demonstrated that early EPCs express the monocyte marker CD14 and hematopoietic marker CD133, but are negative for the progenitor marker CD34 [10]. Some studies found that early EPCs express some typical endothelial markers, such as CD31, vascular endothelial growth factor receptor 2 (VEGFR2), and Tie2; however, mononuclear cells in culture also can acquire these markers through platelet microparticle uptake [2]. Thus, these findings supported the non-endothelial progenitor, mononuclear cell-like nature of early EPCs.

The commonly accepted surface markers expressed by EOCs are CD34 and VEGFR2 [11]. Prasain showed that EOCs express endothelial antigens CD34, VEGFR2, CD146, Flt-1, Flk-Flt-4, and Nrp2, but are negative for the hematopoietic antigens CD45, CD14, CD11b, c-Kit, CXCR4, and CD133 [12]. Thus, although many studies have attempted to identify specific surface markers for these cells, additional studies are still needed [13].

The method commonly used to isolate human EPCs is to plate peripheral blood monocyte fractions in fibronectin-coated dishes, add commercial medium (EGM-2MV), along with fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF). EPCs are then isolated as the cells persisting after 5–7 days exhibit acetylated low-density lipoprotein (ac-LDL) and Ulex europaeus agglutinin I (UEA-1) binding ability [14].

Interestingly, colony-forming assays have recently been developed for isolation of human EPCs. In this method, peripheral blood cells are seeded in fibronectin-coated wells, medium is added, and cell clusters develop within 4–9 days exhibiting a spindle shape; these cells are considered early EPCs. For preparation of EOCs, peripheral blood cells are seeded on collagen-1 coated dishes with appropriate medium and incubated for 2–3 weeks, after which colonies having a cobblestone shape, endothelial surface markers, and capillary-like vessel-forming ability are observed; these cells are considered as EOCs [14].

Additional advancements in isolation methods have been made by various other researchers. For example, Lu et al. demonstrated that density gradient centrifugation combined with differential adherence methods could be used to efficiently isolate EPCs. Moreover, O E et al. demonstrated that nonadhesive, serum-free culture of the CD34-positive human cord blood fraction with a cytokine combination consisting of stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand, interleukin (IL)-3, and basic fibroblast growth factor could promote effective early EPC isolation and expansion [15]. In a previous study, the standard protocol based on the isolation of mononuclear cells from adult peripheral blood was modified by adding a passaging step 7 days after the isolation; this modified protocol resulted in better efficacy in EOC isolation [16]. Furthermore, a study by Tasev et al. suggested that expansion of peripheral blood-derived EOCs in medium supplemented with platelet lysates also permitted generation of sufficient cells [17].

4 Intrinsic properties of early EPCs and EOCs

Early EPCs are intrinsically hematopoietic cells with the molecular profile of pro-angiogenic N2 macrophages and are developed from HSCs. Accordingly, some researchers have suggested the use of circulating angiogenic cells (CACs) to define early EPCs. The majority of early EPCs arise from a CD14-positive subpopulation of peripheral blood mononuclear cells [18] and exhibit a spindle-shaped morphology [19]. Yoder et al. showed that there are two populations when EPCs are cultured; the first can differentiate into macrophages and are spindle-shaped hematopoietic cells that have a mononuclear phenotype, which are defined as early EPCs [20]. Early EPCs do not incorporate into the vasculature of the host but can adhere and accumulate in the surrounding location of impaired vessels loosely [14] in diseased organisms. Notably, however, early EPCs may also participate in inflammatory processes because of their ability to differentiate into macrophages.

EOCs develop from the CD14-negative fraction of peripheral blood mononuclear cells [18] and are directly derived from angioblasts [19], exhibiting a characteristic cobblestone-like morphology [10]. EOCs have robust proliferation, migration, and tube-forming abilities and exhibit relatively high levels of telomerase activity [21]. A study by Yoder et al. confirmed the morphology and characteristics of EOCs in proliferation and tube formation. Additionally, Medina et al. showed that EOCs express transcripts linked to angiogenesis signaling, supporting the endothelial lineage of these cells [22]. Moreover, EOCs also exhibit great homing abilities; once injected into the ventricle, EOCs migrate rapidly to traumatic brain injury zone as early as 3 days after transplantation, which is much more quickly than mature ECs [23]. Over all, EOCs have been shown to possess all the characteristics of true endothelial progenitors [21].

5 Angiogenesis function of early EPCs and EOCs

Early EPCs promote physiological angiogenesis through releasing potent angiogenesis growth factors and cytokines in a paracrine manner. Studies have demonstrated that there are 82 proteins in the conditioned medium of human EPCs. In an experiment, early EPCs were found to affect target cells when separated from them through transwell membranes, demonstrating that early EPCs function in a paracrine manner.

Early EPCs can secrete various angiogenic cytokines, such as IL-8 and VEGF. A study by Katusic demonstrated that early EPCs induce mitogenic effects on vascular endothelial cells through IL-8. Moreover, Yoon et al. showed that early EPCs could augment the angiogenic effects of EOCs through IL-8- and VEGF-dependent mechanisms. Additionally, early EPCs could also enhance the invasiveness of mixed EPCs by upregulating matrix metalloproteinase (MMP)-2 and MMP-9 in response to IL-8 and VEGF treatment [11]. Yoon et al. also demonstrated that early EPCs release large amounts of MMP-9, whose activation results in increased invasiveness [11]. Thus, the secretome of early EPCs can mediate cell–cell interactions and sustain homeostasis in organisms.

Early EPCs can also secrete various chemokines, including stromal cell-derived factor 1 (SDF-1)/CXCR-4. Conditioned medium from EPCs elicits a strong migratory response in ECs *in vitro*, which can be abolished with neutralizing antibodies to SDF-1 [24], revealing the involvement of SDF-1 in the chemotactic function of early EPCs. However, despite extensive studies, additional investigations are needed to elucidate the complete network of factors involved in mediating the paracrine effects of EPCs [25].

EOCs exhibit spontaneous angiogenic effects and can integrate into the systemic vasculature of the host animal, facilitate repopulation of the vasculature through the repair of ECs [26]. Accordingly, EOCs may be promising candidates for cell-based therapy in patients with ischemic diseases. Initial studies have demonstrated that EOCs develop vessels independently. For example, Rong et al. showed that EOCs can develop capillary-like tubes *in situ* spontaneously on Matrigel. In addition, EOCs secrete a few cell factors that can enhance vessel formation. For example, Gulati et al. demonstrated that EOCs express endothelial nitric oxide synthase (eNOS) and caveolin-1 [18], which play important roles in the tube formation ability of EOCs. Yoon et al. showed that EOCs release large amounts of MMP-2, which can mediate the activation of MMP-9, enhance matrix degradation, and augment the invasive capacity of early EPCs [11]. Some studies have also shown that EOCs enhance EPC migration and adhesion through release of SDF-1, CXCL1, macrophage migration inhibitory factor (MIF), and VEGF under hypoxic conditions.

6 Clinical applications of EPCs in neurological diseases

6.1 Relevant mechanisms

Because secreted growth factors and cytokines contribute greatly to endothelial repair, angiogenesis, and the nutrient-rich brain niche [27], the secretome of early EPCs may represent a therapeutic target for the management of stroke. Burlacu et al. showed that the conditioned medium of EPCs is beneficial for EC proliferation [28], indicating that this molecular milieu may have protective effects against stroke.

With regard to EOC-based therapies for stroke, endothelial injury is an important pathophysiological mechanism resulting from associated risk factors, such as hypertension, hyperlipidemia, and diabetes mellitus. Accordingly, one of the main goals of stroke therapy is to repair injured ECs. EOCs can directly differentiate into ECs, increasing the number of migrating ECs in the ischemic area via blood flow. Nih et al. demonstrated that transplanted EPCs and smooth muscle progenitor cells promoted vascular remodeling and angiogenesis in a mouse model of stroke [29].

Vessel thrombosis is another pathophysiological mechanism of stroke. In the treatment of stroke, EOCs may promote neovascularization, enhancing spontaneous angiogenesis combined with new vessel formation from existing capillaries or post-capillary venules. Moreover, the formation of new vessels after EPC transplantation was

found to involve both angiogenesis and vasculogenesis in a mouse model of stroke [30]. Tsuji et al. demonstrated that transplanted EPCs could alleviate brain ischemic injury and transiently augment cerebral blood flow in a mouse model of left middle cerebral artery occlusion (MCAO) [31].

Nerve injury and impaired neurological function occur in animals who experience stroke. EOCs can secrete vessel growth factors to promote the proliferation and migration of neuroblasts from the subventricular zone to the peri-ischemic area, finally differentiating into neurons and oligodendrocytes [32]. Nih et al. demonstrated that transplanted EPCs and smooth muscle progenitor cells can promote sustained neurogenesis and neuroblast migration into infarct areas through mature vessel formation in a mouse model of stroke [29]. In addition, EOCs have also been shown to have anti-inflammatory effects in animals who experience stroke. Jing et al. demonstrated that EOCs have neuroprotective effects on cerebral ischemia/reperfusion injury in rats by blocking cell apoptosis and oxidative stress. The complex mechanisms mediating these effects involve upregulation of Bcl-2 and downregulation of caspase-3, Bax, nuclear factor kappaB (NF- κ B), and malondialdehyde (MDA) in the ischemic penumbra. These changes significantly augment the activity of superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GSH-PX) [33].

Additionally, blood brain barrier (BBB) disruption and neuron death are the pathophysiological mechanism of traumatic brain injury (TBI). Huang et al. found that transplanted EOCs could repair impaired BBB and promote angiogenesis in TBI mice. Among them, the specific mechanism may involve increased expression of tight junction proteins claudin 5 and ZO-1 to improve BBB's integrity [23].

Amyotrophic Lateral Sclerosis (ALS) is a kind of neurodegenerative disease which involves muscle weakness resulting in palsy and death. Stoltz et al. demonstrated that transplanted human cord blood mononuclear cells could delay SOD1G93A mice, a model of familial ALS, symptom progression and promote prolonged survival. The mononuclear cells located in lateral ventricular even 4 months after transplantation but were not found in spinal cord, indicating that mononuclear cells play a beneficial role mainly through developing neuroprotective environment but not cell replacement. They observed that transplanted mononuclear cells secreted lots of anti-inflammatory cytokines and chemokines which could be responsible for function improvement of ALS mouse models [34].

6.2 Clinical applications

Recently, many reports have described the potential clinical applications of EPC-based therapy for stroke. For

example, Moniche et al. infused 3.38×10^6 CD34-positive stem cells intra-arterially into 10 patients with chronic stroke. The results showed that levels of neurotrophic factors were significantly higher than those in control arms, demonstrating that EPCs promote neurogenesis in patients with stroke through secretion of neurotrophic factors. However, no significant neurological functional improvement was observed [35]. Chen et al. administered $3-8 \times 10^6$ CD34-positive stem cells intracerebrally into 15 patients with subacute stroke and showed significant improvements in the National Institutes of Health Stroke Scale and the modified Rankin scale compared with the control group [36]. Prasad et al. injected 2.8×10^8 bone marrow mononuclear cells intravenously into 58 patients with chronic stroke; however, no significant neurological function recovery was observed compared with the control arm, and there were no treatment-related adverse effects [37]. Further studies are needed to optimize conditions for EPC-based therapy, including the appropriate cell origin, cell dose, injection method, intervention time, and recipient type. Additional large-scale clinical studies are needed to confirm the efficacy and safety of this cell-based therapy.

Besides, Peleteiro et al. demonstrated that intracerebral hemorrhage patients with good outcome showed statistically higher EPCs numbers on day 3 and day 7. Among them, numbers of EPCs on day 7 were independently associated with good functional outcome and were negatively associated with residual volume [38]. Chung et al.'s study demonstrated that EPCs could modulate healthy subjects' cerebral vasoreactivity, indicating that EPCs could be used therapeutically and for prevention in conditions with cerebral endothelial impairment and cerebral ischemia [39].

Li Liu et al. showed that for TBI patients the level of EPCs was suppressed 24–48 h after injury, and then rapidly increased, reaching the highest on day 5–7 post-trauma. Circulating EPC level of patients with function improvement were significantly higher than those with deteriorated condition, and remained persistently low in patients died of trauma, indicating that level of circulating EPCs correlated with clinical outcome of TBI patients and may offer as a potential prognostic marker for TBI [40].

7 Summary

Putative EPCs are a heterogeneous population. An overview of the properties and functional characteristics of early EPCs and EOCs are respectively shown in Table 1 and Table 2. Early EPCs have low proliferation and colony-forming abilities and have been shown to release numerous cytokines, promoting angiogenesis, nerve repair, and EC repair in a paracrine manner. EOCs have high proliferative potential and colony-forming ability.

Table 1 Biological properties of early EPCs and EOCs

Biological properties	EOCs	Early EPCs
Time for culture growth	7–21 days	3–5 days
Morphology	Confluent cobblestone monolayer	Round to spindle-shaped
Surface markers	CD34+ VEGFR-2+ CD45– CD14– CD133–	CD34 ^{low} VEGFR-2 ^{low} CD45+ CD133+
Gene characteristics	Related to angiogenesis signaling pathways	Related to immunity and inflammation
Related transcription factors	GATA6/IRF6	MAF/MAFB
Proteomic analysis	90% of spots are common with endothelial cells	77% of spots are common with monocytes
Specific properties	Bind UEA-I lectin and take up LDL	Bind UEA-I lectin and take up LDL

UEA-I Ulex europaeus agglutinin I; *LDL* low-density lipoprotein

Table 2 Functional characteristics of early EPCs and EOCs

Functional characteristics	EOCs	Early EPCs
De novo tube formation	Generate de novo tubes in Matrigel	None
Cytokine secretion	Low MMP-2	High MMP-9 CXCR4 VEGF, IL-8 VEGF, HGF, G-CSF
Nitric oxide production	High	Low
eNOS and caveolin-1	Express	None
Anti-platelet aggregation	Better	Good
Pro-inflammation	None	Present

VEGFR-2 vascular endothelial growth factor receptor 2; *eNOS* endothelial nitric oxide synthase

Moreover, EOCs promote vessel formation by displaying spontaneous angiogenesis ability, integrating into the systemic vasculature of the host animal, and remodeling ECs. EOCs possess all characteristics of true endothelial progenitors and have advantages in EPC-based therapies for ischemic diseases. A number of preclinical and clinical applications of EPCs in neurological diseases are under study. However, more studies are needed to determine the specific characteristics of EPCs and the relevant mechanisms of EPCs for neurological diseases.

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Compliance with ethical standards

Conflicts of interest None.

Ethical statements All descriptions were based on previous published studies, thus no ethical approval and patient consent are required.

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