British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01857.x
www.brjpharmacol.org

Themed Section:
Regenerative Medicine and Pharmacology: A Look to the Future

REVIEW

Revisiting cardiovascular regeneration with bone marrow-derived angiogenic and vasculogenic cells

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Keywords
cell therapy; cardiovascular disease; bone marrow; haematopoietic; paracrine; transdifferentiation; CD31; neovascularization; angiogenesis; vasculogenesis

Received
7 November 2011
Revised
18 December 2011
Accepted
5 January 2012

Cell-based therapy has emerged as a promising therapy for cardiovascular disease. Particularly, bone marrow (BM)-derived cells have been most extensively investigated and have shown encouraging results in preclinical studies. Clinical trials, however, have demonstrated split results in post-myocardial infarction cardiac repair. Mechanistically, transdifferentiation of BM-derived cells into cardiovascular tissue demonstrated by earlier studies is now known to play a minor role in functional recovery, and humoral and paracrine effects turned out to be main mechanisms responsible for tissue regeneration and functional recovery. With this advancement in the mechanistic insight of BM-derived cells, new efforts have been made to identify cell population, which can be readily isolated and obtained in sufficient quantity without mobilization and have higher therapeutic potential. Recently, haematopoietic $CD31^+$ cells, which are more prevalent in bone marrow and peripheral blood, have been revealed to have angiogenic and vasculogenic activities and strong potential for therapeutic neovascularization in ischaemic tissues. This article will cover the recent advances in BM-derived cell-based therapy and implication of $CD31^+$ cells.

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Abbreviations
Ang-1, angiopoietin-1; bFGF, basic fibroblast growth factor; BM, bone marrow; CVD, cardiovascular disease; CXCR4, C-X-C chemokine receptor 4; EC, endothelial cell; ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell; ESC, embryonic stem cell; GATA2, GATA binding protein-2; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; HBEGF, heparin binding EGF-like growth factor; HGF, hepatocyte growth factor; HPC, haematopoietic progenitor cell; HSC, haematopoietic stem cell; HUVEC, human umbilical vein endothelial cells; IFN, interferon; IGF, insulin-like growth factor; IHD, ischaemic heart disease; iPSC, induced pluripotent stem cell; Kit, tyrosine protein kinase Kit; lin, lineage; MCP-1, monocyte chemotactic protein-1; MI, myocardial infarction; MNC, mononuclear cell; MSC, mesenchymal stem cell; NRP-1, neuropilin-1; PB, peripheral blood; PIGF, placental growth factor; SDF-1, stromal derived factor-1; Sfrp2, secreted frizzled related protein 2; Thy-1, thymocyte differentiation antigen 1; Tie 2, tyrosine kinase with immunoglobulin-like and EGF-like domains 2; VE-cad, vascular endothelial cadherin

Introduction

Ischaemic cardiovascular disease is the leading cause of death in the United States (Roger *et al.*, 2010). There are many pharmacological and surgical therapies commonly used to treat patients with cardiovascular diseases. However, a majority of patients show deteriorated symptoms, progressive heart failure and increasing need for hospitalization (Fang *et al.*,

2008; Murry and Keller, 2008). Moreover, limited available therapies and need for multiple treatments are problems for tackling peripheral vascular disease (Taylor *et al.*, 2007; Feinglass *et al.*, 2009) in addition to the risk of amputation. Therefore, there has been increasing demand for new therapies for ischaemic cardiovascular diseases. The main cause of these diseases is atherosclerosis which leads to blocking of the blood vessels. Once vessels are blocked, new vascular growth is required to rescue the endangered target tissues. As endothelial cells (ECs) are a major component of blood vessels and a leading component of vascular growth, therapies for treating ischaemic cardiovascular diseases should target regeneration of ECs.

Stem cells have drawn people's attention over the past decade due to their potential for new tissue generation. Embryonic stem cells (ESCs) have a strong regenerative potential and thus have been widely studied for cardiovascular regeneration in animal models. However, the risks of teratoma formation (Cao *et al.*, 2006) and host immune response to allogeneic ESCs (Bifari *et al.*, 2010) as well as ethical concerns need to be solved for clinical translation. The recent discovery of induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007) offered new hope to circumvent ethical and immunologic concerns associated with ESCs. However, many questions still remain in regard to their therapeutic effects and adverse effects (Hacein-Bey-Abina *et al.*, 2003; Dhodapkar *et al.*, 2010; Hu *et al.*, 2010; Kim *et al.*, 2010b; Polo *et al.*, 2010; Zhao *et al.*, 2011).

Although seemingly not as potent as ESCs, adult stem or progenitor cells opened the cell therapy era for treating cardiovascular diseases. Among many types of adult stem or progenitor cells, bone marrow (BM)-derived cells have been most widely investigated and shown tissue regenerative effects in preclinical studies as well as some clinical trials. Particularly, BM-derived endothelial progenitor cells (EPCs) have been studied by many research groups and have shown therapeutic potentials in animal models of myocardial and peripheral vascular ischaemia (Kalka *et al.*, 2000; Murohara *et al.*, 2000; Schatteman *et al.*, 2000; Kawamoto *et al.*, 2001; 2006; Kocher *et al.*, 2001; Iwasaki *et al.*, 2006; Jeong *et al.*, 2009). Moreover, a series of clinical trials using EPCs or similar BM cells has shown therapeutic benefits to treat post-myocardial infarction (MI) cardiac dysfunction and critical limb ischaemia (Assmus *et al.*, 2002; 2006; Bartunek *et al.*, 2005; Boyle *et al.*, 2006; Schaefer *et al.*, 2006; Li *et al.*, 2007; Losordo *et al.*, 2007; 2011; Stamm *et al.*, 2007; Tatsumi *et al.*, 2007; Hare *et al.*, 2009; Burt *et al.*, 2010; Kuroda *et al.*, 2011; Miettinen *et al.*, 2010). On the other hand, other studies have revealed conflicting results with regard to the effects of BM-derived mononuclear cells (MNCs) on myocardial ischaemia in a similar subset of patients (Janssens *et al.*, 2006; Lunde *et al.*, 2006; Meyer *et al.*, 2006; Penicka *et al.*, 2007). As there are larger clinical trials with MNCs, early EPCs or $CD34^+$ cells are under way, and more agreeable conclusion will be drawn in the near future. In addition, there have been new advances in the understanding of mechanisms governing therapeutic effects of BM-derived stem and progenitor cells. Earlier studies demonstrated that transdifferentiation into vasculature and cardiomyocytes is the dominant mechanism responsible for cardiac regeneration or repair (Asahara *et al.*, 1999; Jackson *et al.*, 2001; Kocher *et al.*, 2001; Orlic *et al.*, 2001; Murayama *et al.*, 2002; Yeh *et al.*, 2003; Ii *et al.*, 2005; Bauer *et al.*, 2006; Iwakura *et al.*, 2006; Iwasaki *et al.*, 2006; Kawamoto *et al.*, 2006; Masuda *et al.*, 2007). However, later studies have uncovered that this transdifferentiation potential was overestimated (Balsam *et al.*, 2004; Murry *et al.*, 2004; Ziegelhoeffer *et al.*, 2004) and humoral or paracrine effects are the main mechanism underlying therapeutic potential of BM-derived cells. These humoral mechanisms, by protecting ongoing cell apoptosis and degeneration, inducing neovascularization, and promoting regeneration of endothelial cells, cardiomyocytes and smooth muscle cells through soluble factors or cell-to-cell contact (Rehman *et al.*, 2003; Kinnaird *et al.*, 2004; Gnecchi *et al.*, 2005; Ii *et al.*, 2005; Urbich *et al.*, 2005; Yoon *et al.*, 2005b; Uemura *et al.*, 2006; Cho *et al.*, 2007; Miyamoto *et al.*, 2007; Dai *et al.*, 2008), can benefit regeneration and repair of myocardium or peripheral vascular tissues. There have been attempts to identify soluble factors responsible for paracrine effects of mesenchymal stem cells (MSCs), which led to the identification of secreted frizzled related protein 2 (Sfrp2), an antagonist of the Wnt signalling (Mirotsou *et al.*, 2007). In this study, Sfrp2 was shown to have dose-dependent cytoprotective effects with concentration of up to 15 nM in *in vitro* caspase activity assay. In addition, a follow-up study demonstrated that exogenous delivery of Sfrp2 to rat hearts at a therapeutic dose of 4 μg per heart improved cardiac function in experimental MI (He *et al.*, 2010).

Based on this new discovery on mechanisms of BM-derived cells, there have been efforts to identify 'effector' cells based on markers that are not specifically restricted to stem or progenitor cells and to utilize such cells for therapeutic neovascularization (Hur *et al.*, 2007; Kim *et al.*, 2010a,c). In fact, a subpopulation of BM- and peripheral blood (PB)-derived MNCs expressing CD31 on the surface was shown to have higher angiogenic and vasculogenic activities and exert efficient neovascularization in hindlimb ischaemia (Kim *et al.*, 2010a,c). Hence, this review will cover the characteristics and the therapeutic potential of EPCs, BM-MNCs and recently identified $CD31^+$ cells.

Endothelial progenitor cell

The EC population has been studied based on the idea that these cells may play important roles in maintaining vascular homeostasis and in the pathogenesis of a variety of disease. Asahara and colleagues first demonstrated the presence of BM-derived circulating progenitor cells or angioblasts in human peripheral blood (hPB) (Asahara *et al.*, 1997). These progenitor cells displayed EC properties but also had the potential to differentiate into ECs. These cells were referred to as EPCs. This seminal publication manifested a novel concept of post-natal vasculogenesis by postulating that, in addition to vessel wall ECs, BM-derived circulating progenitor cells participate in blood vessel growth, maintenance and repair. This population was shown to be incorporated into the vasculature in adult animals and to induce new vessel formation in ischaemic tissues (Asahara *et al.*, 1999). The transplantation of EPCs into ischaemic tissues induced neovascularization and helped regenerate ischaemic tissue damage (Kalka

et al., 2000). Although this novel concept of post-natal vasculogenesis has been widely accepted, the precise identification of genuine EPCs has been complicated by the lack of specific markers and phenotype diversity. In addition, the advance of technologies allowed to identify the role of EPCs in disease pathogenesis (Bertolini *et al.*, 2006; 2007; Dimmeler *et al.*, 2008; Kawamoto and Losordo, 2008) besides a normal component of the formed elements of circulating blood (Schatteman *et al.*, 2007).

Early EPC

Initially, CD34 or VEGF receptor (VEGFR)-2, which was already applied in the haematopoietic stem or progenitor cell isolation, was used to isolate circulating EPCs or putative angioblasts from PB (Asahara *et al.*, 1997; Shi *et al.*, 1998; Peichev *et al.*, 2000). However, due to the lack of the specific surface markers for identifying circulating EPCs, EPCs were enriched by short-term culture of various BM cell fractions in endothelial differentiation media. For example, CD133, which is displayed on immature haematopoietic stem cells (HSCs), was used for culture derivation of EPCs (Fernandez Pujol *et al.*, 2000). For therapeutic purposes, short-term culture of MNCs was widely used for deriving EPCs. In this case, entire MNCs were cultured for 4–7 days on vitronectin- or fibronectin-coated dishes and adherent cells were used as EPCs although these cells are not EPCs as a whole but EPC-enriched cells (Asahara *et al.*, 1997; 1999; Dimmeler and Zeiher, 2000; Kalka *et al.*, 2000; Dimmeler *et al.*, 2001). Typically, a majority of these cultured cells displayed endothelial-like characteristics represented by the uptake of acetylated low-density lipoproteins and the binding of lectins and expression of several EC-specific proteins [VEGFR-2, Tie2, vascular endothelial (VE)-cadherin, von Willebrand factor, endothelial NOS (eNOS) and CD146] and showed a low proliferation rate. However, other studies have raised questions regarding the endothelial-like features of these EPCs by showing that these cells also express monocyte/macrophage markers such as CD45, CD14, CD11b and CD11c (Schmeisser *et al.*, 2001; Gulati *et al.*, 2003; Rehman *et al.*, 2003; Ingram *et al.*, 2005). More recently, these cells were referred to as circulating angiogenic cells (Gulati *et al.*, 2003) as these EPCs rarely give rise to ECs *in vivo* but contribute to vessel formation mainly through their angiogenic effects. Alternative techniques have been used to isolate cells similar to these EPCs, where whole MNCs were seeded on fibronectin-coated plates. After 2 days, only non-adherent cells were collected for removal of mature ECs and macrophages, and subsequently re-seeded on fibronectin-coated plates. Colonies were generated after 5–9 days and named colony-forming unit-Hill or colony-forming unit-ECs (Hill *et al.*, 2003). However, the identity and characteristics of these cells appear not to be similar to aforementioned early EPCs.

EPCs contribute to vascular regeneration, in part, by incorporating into the neovasculature and differentiating into ECs (Asahara *et al.*, 1997; 1999; Kocher *et al.*, 2001). Evidence also suggests that populations of cells containing EPCs are inherently multipotent and may also include smooth muscle progenitor cells (Wang *et al.*, 2003). EPC differentiation into cardiomyocytes and smooth muscle cells has been reported in mouse (Yeh *et al.*, 2003) and rat (Iwasaki *et al.*, 2006; Kawamoto *et al.*, 2006) models of MI, and PB EPCs mobilized by granulocyte-colony stimulating factor (G-CSF) administration and myocardial ischaemia have been shown to express cardiac-, muscle-, liver- and neural-lineage markers (Kucia *et al.*, 2004; Ratajczak *et al.*, 2004). Most reports have found little evidence of fusion between EPCs and cells of other lineages (Koyanagi *et al.*, 2005; Iwasaki *et al.*, 2006); however, results from one study indicated that 70% of newly formed cardiomyocytes in mice administered human EPCs contained both human and mouse X chromosomes, suggesting that EPCs may transdifferentiate by fusing with cells of a different lineage (Zhang *et al.*, 2004). The proportion of ECs displaying evidence of human–mouse cell fusion was less than 3%. In fact, most recent studies have suggested that although transdifferentiation of EPCs are possible, such phenomenon is not prevalent and may not underlie biologic effects derived from EPCs. In addition to their role as a structural tissue component, EPCs express many factors that contribute to tissue regeneration and preservation, including the eNOS and inducible iNOS, which increase circulation by dilating capillaries (Ii *et al.*, 2005), and pro-angiogenic or anti-apoptotic growth factors [e.g. VEGF, hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1)] (Urbich *et al.*, 2005). Growth factor release stimulates the recruitment of additional EPCs to the ischaemic area (Cho *et al.*, 2007) and influences the proliferation, migration and survival of both EPCs and pre-existing mature ECs (Folkman, 1995). The therapeutic potential has been evaluated in many studies. Cells were often administered systemically in early investigations, but more recent experiments have tended to employ cell transplantation (i.e. injection directly into the infarcted artery or ischaemic tissue) in an effort to overcome the low rate of retention by systemically administered cells. Transplantation of EPCs (Kalka *et al.*, 2000; Kawamoto *et al.*, 2001; 2003; Kocher *et al.*, 2001; Murohara, 2001) augmented neovascularization in animal models of acute myocardial ischaemia, and evidence of similar effects in humans has also been reported (Assmus *et al.*, 2002; Stamm *et al.*, 2003; Dobert *et al.*, 2004).

Late EPC

Other types of EPCs have been discovered from circulating MNCs, such as outgrowth ECs (Lin *et al.*, 2000), late EPCs (Shi *et al.*, 1998) or endothelial colony-forming cells (ECFCs) as these cells appear late (typically more than 2 weeks) in the conventional EPC culture conditions. Although the culture methods are somewhat variable, these cells essentially share common characteristics in cell morphology (round), proliferation rate (rapid) and surface marker expression (EC markers only) (Ingram *et al.*, 2004; 2005). Specifically, ECFCs do not express haematopoietic (CD45) and monocytic (CD14) markers but express most EC proteins. However, paracrine effects were limited compared with early EPCs and their vasculogenic effects were only demonstrated in a Matrigel™ plug assay (Ingram *et al.*, 2004; Yoon *et al.*, 2005a). Thus, their EC generation capabilities and regenerative or therapeutic effects on vasculature in ischaemic animal models need to be further investigated. It is possible that these cells are primitive circulating ECs or ECs sloughed off from vessels. The difference between ECFCs and mature ECs also remains to be

determined. For therapeutic application, the clinically compatible culture systems need to be established.

Haematopoietic stem cells

HSCs are multipotent stem cells that give rise to all the blood cell types such as the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells), and have the capacity for self-renewal (Reya *et al.*, 2001). In adult vertebrates, HSCs originate from BM and occupy <1% of total BM cells. The haematopoietic tissue contains cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent and unipotent progenitors. HSCs constitute 1:10 000 of cells in myeloid tissue. A fraction of HSCs are also found in circulating PB. HSCs are one of the best characterized stem cells and identified by a lack of markers that are used for detection of lineage commitment (lin^-) and the presence of combinations of the following markers: CD133, CD34, CD38, CD90 (Thy1), CD105 and CD117 (C-kit) (Spangrude *et al.*, 1988; Baum *et al.*, 1992; Seita and Weissman, 2010).

HSCs have been used for the treatment of haematologic disease patients for more than three decades. The transplantation of a single HSC can constitute all haematopoietic cells in an organism and fulfil the criteria of every stem cell function. Due to their multipotency, in earlier studies, HSCs were used to regenerate damaged myocardium (Orlic *et al.*, 2001). This study has shown that HSCs could generate new cardiomyocytes, ECs and smooth muscle cells, and improve post-MI cardiac function. Another versatile capacity of HSCs has been reported with side population cells, which expel Hoechst dye (Goodell *et al.*, 1996). The differentiation potentials of these cells into cardiomyocytes, ECs and vascular smooth muscle cells were also demonstrated in an MI model (Jackson *et al.*, 2001). However, other studies have argued that HSCs represented by lin-negative, sca1-positive and c-kit-positive cells do not transdifferentiate into any cardiovascular cells in the infarcted heart after HSC transplantation (Balsam *et al.*, 2004; Murry *et al.*, 2004). These studies were performed in murine HSCs and no such experiments were conducted with specific human HSCs other than $CD34^+$ cells and $CD133^+$ cells that are enriched with human HSCs and EPCs. Studies using human $CD34^+$ cells (Yeh *et al.*, 2003; Kawamoto *et al.*, 2006; Losordo *et al.*, 2007; Wang *et al.*, 2010) or $CD133^+$ cells (Leor *et al.*, 2006; Schots *et al.*, 2007; Flores-Ramirez *et al.*, 2010) have been reported to improve post-MI cardiac function; however, the therapeutic mechanism turned out to be non-transdifferentiation effects. As $CD34^+$ cells or $CD133^+$ cells are present in low numbers in circulation, the use of mobilizing cytokines such as G-CSF for obtaining a large number of cells needed for clinical use incurs high costs and risk to patients.

BM-mononuclear cells

BM-mononuclear cells (BM-MNCs) comprise a mixed population of cells (e.g. haematopoietic cells, fibroblasts, osteoblasts, myogenic cells, endothelial-lineage cells) (Kamihata *et al.*, 2001). Transplantation of BM-MNCs (Fuchs *et al.*, 2001; Kamihata *et al.*, 2001) augmented neovascularization in animal models of acute myocardial ischaemia, and evidence of similar effects in humans has also been reported (Strauer *et al.*, 2002; Tateishi-Yuyama *et al.*, 2002; Perin *et al.*, 2003; Mocini *et al.*, 2006). The monocyte/macrophage fraction of BM-MNCs expresses important angiogenic growth factors and cytokines [e.g. VEGF, basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), IL-1β, TNF-α] and stimulates angiogenesis (Leibovich *et al.*, 1987; Giulian *et al.*, 1988; Kamihata *et al.*, 2001). This cocktail of angiogenic factors may also put forth potent paracrine effects that enhance the proliferation, homing and differentiation of resident stem cells (Toda *et al.*, 2003).

The various effects exerted by the many cell types in the BM-MNC population are not exclusively beneficial. Lymphocytes and monocytes/macrophages are the most prevalent MNCs and pro-inflammatory cytokines produced by these cells including IL-1, IL-2, IL-6, IL-12, IFN-γ, lymphotoxin and TNFs (Mosmann, 1994; O'Garra and Murphy, 1996; Chandrasekar *et al.*, 1999) may have a negative inotropic effect in the myocardium. In addition, the high number of inflammatory cells present in the MNC population may accelerate myocardial damage after MI, and severe haemorrhaging or inflammation caused by transplanted MNCs could interfere with the long-term survival and differentiation of the transplanted cells (Kawamoto *et al.*, 2006).

Non-transdifferentiation therapeutic effects of BM-derived cells

Despite the fact that BM-derived stem or progenitor cells have emerged as a promising therapeutic modality, there have been several controversies regarding therapeutic mechanisms for ischaemic diseases. In particular, the plasticity or true tissue generation capacity (transdifferentiation into cardiomyocytes, smooth muscle cells and ECs) of BM-derived cells has been widely debated (Balsam *et al.*, 2004; Murry *et al.*, 2004; Ziegelhoeffer *et al.*, 2004). Although therapeutic effects are observed, it is now well accepted that the occurrence of vasculogenesis and exogenous myogenesis in damaged tissues are very low. Many recent studies suggested that the paracrine mechanism could be a major mechanism to mediate therapeutic effects in cardiovascular diseases. BM cells release angiogenic factors such as VEGF, monocyte chemotactic protein-1 (MCP-1, also known as CCL2), FGF-2, Ang-1 and Wnt (Kamihata *et al.*, 2001; Fuchs *et al.*, 2005; Barcelos *et al.*, 2009) and cultured early EPCs secrete HGF, VEGF, G-CSF, IGF-1 and stromal derived factor-1 (SDF-1) (Rehman *et al.*, 2003; Urbich *et al.*, 2005). Rehman *et al.* (2003) quantified various growth factors released from cultured early EPCs to the culture media over 72 h: VEGF (7601 ± 2611 pg), HGF (6912 ± 1345 pg), G-CSF (8925 ± 3255 pg) and GM-CSF (492 ± 453 pg) per 10^6 cells. Kinnaird *et al.* also measured VEGF (375 $pg \cdot \mu g^{-1}$ protein), bFGF (2320 $pg \cdot \mu g^{-1}$), placental growth factor (119 $pg \cdot \mu g^{-1}$) and MCP-1 (150 $pg \cdot \mu g^{-1}$) secreted from MSCs into the culture media for 24 h (Kinnaird *et al.*, 2004). Urbich *et al.* also reported that

cultured early EPCs released significantly higher amount of pro-angiogenic factors VEGF and SDF-1, IGF-1 and HGF compared to human umbilical vein endothelial cells determined by ELISA of culture media (Urbich *et al.*, 2005). One recent study reported an important role of Wnt signalling in mediating angiogenic effects of human fetal CD133$^+$ cells on ischaemic wounds (Barcelos *et al.*, 2009). These growth factors promote angiogenesis, protect against tissue apoptosis or necrosis, and induce endogenous resident stem cell migration and proliferation through a paracrine response (Kinnaird *et al.*, 2004; Gnecchi *et al.*, 2005; Urbich *et al.*, 2005; Yoon *et al.*, 2005b; Tolar *et al.*, 2007; Barcelos *et al.*, 2009). Additionally, it is now known that these humoral effects are not only attributed to implanted cells, but also to host tissues that had received cell therapy (Tateno *et al.*, 2006; Tolar *et al.*, 2007). However, the evidence of paracrine effects derived from late EPCs or ECFCs is not clear. As a mechanism for transdifferentiation, studies have claimed that fusion can be a mechanism for transdifferentiation (Terada *et al.*, 2002; Ying *et al.*, 2002). Although earlier reports emphasized the role of fusion for transdifferentiation (Alvarez-Dolado *et al.*, 2003; Nygren *et al.*, 2004), more recent studies have shown that fusion is only partly or minimally responsible for the phenotypic changes of stem cells (Yoon *et al.*, 2005b).

Haematopoietic CD31$^+$ cells

Role of CD31 in vascular biology

CD31, also known as platelet endothelial cell adhesion molecule-1, is a 130 kDa transmembrane protein consisting of six extracellular immunoglobulin folds (Figure 1). In its cytoplasmic domain, there are two immunoreceptor tyrosine-based inhibitory motifs (ITIM) for interactions with signalling molecules. CD31 is expressed on the cell surface of ECs and haematopoietic cells such as monocytes, platelets, neutrophils, natural killer cells, megakaryocytes and some T cells. CD31 mediates homotypic adhesion between adjacent ECs as well as between ECs and leukocytes (Albelda *et al.*, 1991; Xie and Muller, 1993). A role of CD31 in migration through ECs has also been reported for neutrophils and monocytes (Muller *et al.*, 1993), and subsequently for numerous other cell types including natural killer cells (Berman *et al.*, 1996), haematopoietic progenitor cells (HPCs) (Voermans *et al.*, 2000) and certain subsets of lymphocytes (Zocchi *et al.*, 1996; Schenkel *et al.*, 2004).

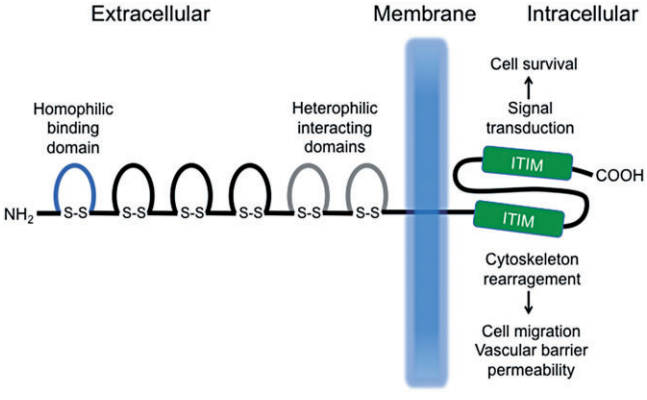

Figure 1

The structure of CD31. CD31 is a 130-kD type I transmembrane glycoprotein and a member of Ig gene superfamily.

Role of CD31 in survival and angiogenesis

Cell–cell and cell–extracellular matrix interactions have been shown to play pivotal roles in coordinating EC proliferation and apoptosis required for proper blood vessel formation and regression. There is growing evidence that CD31 could transduce signals that suppress cell death. It has been proposed that homophilic interactions of CD31 between ECs and monocytes decrease apoptotic EC death (Noble *et al.*, 1999). This finding suggests that CD31 homophilic interactions result in the transmission of pro-survival signals. In addition, CD31 engagement has been reported to induce Akt, a serine/threonine protein kinase, phosphorylation. CD31 itself has also been involved during EC apoptosis by two pathways: a metalloproteinase-dependent cleavage and a caspase-mediated cleavage of the cytoplasmic tail (Ilan *et al.*, 2001).

Given its abundant expression in ECs, CD31 has been shown to be involved in the initial stabilization and formation of cell–cell contacts at lateral junctions of ECs, the maintenance of a vascular permeability barrier, modulation of cell migration, transendothelial migration of monocytes and neutrophils, and formation of blood vessels in angiogenesis (Newman *et al.*, 1990; Albelda *et al.*, 1991; Muller *et al.*, 1993; DeLisser *et al.*, 1997). Additionally, CD31 was found to form a functional complex with VE-cadherin, β-catenin and F-actin to control EC tube formation (Matsumura *et al.*, 1997). More recent data showed the involvement of CD31 in the adhesion/signalling events necessary for the migration of ECs and subsequent tube formation during angiogenesis, independent of VE-cadherin (Cao *et al.*, 2002).

Identification of specialized multimodal angio-vasculogenic cells: role of haematopoietic CD31$^+$ cells

As the major mechanisms underlying therapeutic effects for BM-derived stem or progenitor cells turned out to be humoral effects (Kinnaird *et al.*, 2004; Gnecchi *et al.*, 2005; Uemura *et al.*, 2006; Cho *et al.*, 2007), identifying such cells could lead to the development of next generation cell therapy. Therefore, recent studies explored whether or not cells enriched with humoral activities can be found from BM and/or PB (Kim *et al.*, 2010a,c). At the same time, these studies sought to use a surface marker to isolate these cells. By avoiding cell culture, direct isolation using a surface marker can circumvent deleterious effects associated with the use of animal serum and extra costs related to cell cultivation. Since CD31 is a well-known EC marker, CD31$^+$ cells may meet such end. In fact, these two studies demonstrated more broadly that BM-derived CD31$^+$ cells have multimodal effects including angiogenic, vasculogenic, higher adhesion and almost exclusive haematopoietic stem/progenitor cell activities (Figure 2).

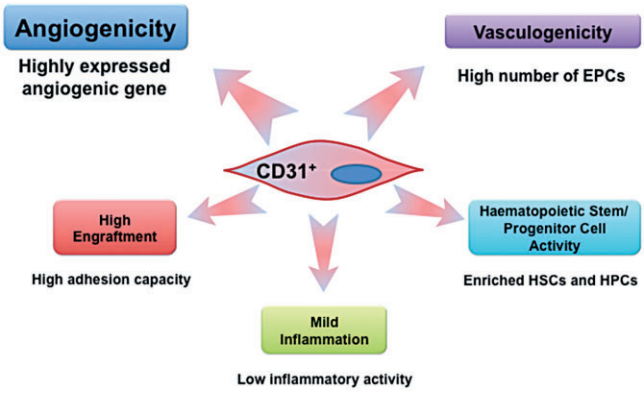

Figure 2

Pleiotropic effects of BM-derived haematopoietic CD31$^+$ cells. Various beneficial effects of CD31$^+$ cells on therapeutic neovascularization and tissue regeneration are summarized.

Angiogenic activities

This study first looked at the angiogenic activities of the CD31$^+$ cells. Genome-wide gene expression studies followed by gene set enrichment analysis and hierarchical cluster analysis with mouse BM (mBM), human BM (hBM) and hPB showed that angiogenic genes were globally up-regualted in CD31$^+$ cells compared with CD31$^-$ cells (Kim *et al.*, 2010a,c). The angiogenic genes were significantly enriched in the CD31$^+$ cells compared with the corresponding CD31$^-$ cells. mBM-CD31$^+$ cells expressed very high levels of Ang-1 and GATA2 compared with the CD31$^-$ cells, whereas hBM-CD31$^+$ cells expressed higher levels of heparin-binding EGF-like growth factor (HBEGF) and IL-8 than hBM-CD31$^-$ cells. Ang-1 is a well-known angiogenic growth factor (Jones *et al.*, 2001; Cho *et al.*, 2004) and GATA2 is a transcription factor, which, when activated, increases angiogenesis (Mammoto *et al.*, 2009). HBEGF is involved in the recruitment of vascular smooth muscle cells (Iivanainen *et al.*, 2003) and IL-8 is a macrophage-derived mediator of angiogenesis (Koch *et al.*, 1992). Neuropilin-1 (NRP1) is the most highly expressed angiogenic gene in hPB-CD31$^+$ cells compared with hPB-CD31$^-$ cells. NRP1 is required for vascular development and mediates VEGF-dependent angiogenesis (Lee *et al.*, 2002).

Vasculogenic properties

One important question is to address whether any haematopoietic cells contribute to true EC generation and to determine the identity of such cells. Prior studies from other investigators and ours have provided evidence that there is quite a reasonable possibility that certain BM-derived cell populations can give rise to ECs (Shi *et al.*, 1998; Lyden *et al.*, 2001; Orlic *et al.*, 2001). However, some earlier studies have strongly argued against these phenomena (Balsam *et al.*, 2004; Ziegelhoeffer *et al.*, 2004; O'Neill *et al.*, 2005). Representative studies that refuted EC generation by haematopoietic cells used MI models (Balsam *et al.*, 2004; Murry *et al.*, 2004) to prove the vasculogenic effects. However, an MI model allows very minimal engraftment of any cells, not only BM-derived cells. Studies have shown that the engraftment rate of embryonic stem cells differentiated into cardiomyogenic lineages, or cardiac stem or progenitor cells are also very minimal in an MI model (Smith *et al.*, 2007; Smits *et al.*, 2009; Tang *et al.*, 2010). The constant motion of the heart, ongoing inflammation in acute stages of infarction, and little oxygen and nutrients in the acutely ischaemic heart put the implanted cells into an environment too harsh for engraftment and survival. Tight junctions between cardiomyocytes could be another hurdle for engraftment of externally injected cells. Furthermore, most studies refute the transdifferentiation potential based on extremely low rate of cardiomyocyte transdifferentiation from BM cells rather than ECs. In fact, one study by the same author showed that EC transdifferentiation is not infrequent in transplanted heart samples (Virag and Murry, 2003). Thus, it appears the transdifferentiation potential of BM cells into ECs should be re-evaluated. Amid such controversy, independent studies have shown such potential that if appropriate BM-derived cells are injected into more permissive models, they can give rise to ECs. Particularly, studies using tumour models have clearly demonstrated transdifferentiation potential of BM cells into ECs (Nolan *et al.*, 2007; Gao *et al.*, 2008). Together, these studies suggest that the generation of ECs from BM or PB cells may not only depend on the cell types but also the experimental models or host environment.

The vasculogenic effects of CD31$^+$ cells have been investigated in recent studies (Kim *et al.*, 2010a,c). *In vitro* assays showed that mBM-, hBM- and hPB-CD31$^+$ cells generated a markedly high number of EPCs under culture, compared with the CD31$^-$ cells. Results from an *in vitro* EC differentiation assay using hPB-CD31$^+$ cells showed expression of EC-specific markers, such as von Willebrand factor, VEGFR-2, VE-cadherin and CD31, in CD31$^+$ cells. Intriguing morphological changes of the hPB-CD31$^+$ cells were observed in this culture. Under endothelial differentiation conditions, hPB-CD31$^+$ cells formed cellular aggregates on day 7, which subsequently underwent tubular structural changes within the round cell cluster by day 10 followed by formation of complete linear tubular structures that mimicked *in vivo* vasculogenesis. These tubular structures stained positive for lectin and took up acetylated human low-density lipoprotein, indicating EC characteristics. Further evidence of vasculogenesis was shown by *in vivo* animal studies. A mouse model of hindlimb ischaemia was used for testing vasculogenic activities of CD31$^+$ cells (Kim *et al.*, 2010a,c). Given the controversy of transdifferentiation potential of BM cells (Lyden *et al.*, 2001; Rehman *et al.*, 2003; Balsam *et al.*, 2004; Ziegelhoeffer *et al.*, 2004; Nolan *et al.*, 2007; Gao *et al.*, 2008), a series of rigorous methods and criteria were used to confirm differentiation. Confocal microscopy with 3D reconstruction of multiple images was used as a first step. This technique clearly demonstrated that a fraction of CD31$^+$ cells was co-localized with ECs within the vascular structure even up to 8 weeks after. In addition, flow cytometric analysis of enzymatically digested hindlimb tissues showed that up to 4% of the ECs in the ischaemic tissues was derived from transplanted mBM- or hPB-CD31$^+$ cells. Fluorescent *in situ* hybridization of the digested tissues further confirmed the contribution of hPB-CD31$^+$ cells into ECs (Kim *et al.*, 2010c). No other studies adopted all of these technologies to prove transdifferentiation of haematopoietic cells. These data clearly indicate that

functional ECs can be derived from directly injected CD31$^+$ cells in the ischaemic tissue.

Higher adhesion and engraftment potential

Studies have shown that engraftment of transplanted neonatal cardiomyocytes is less than 25% within 24 h of MI in animal models (Muller-Ehmsen *et al.*, 2002) and 5% after 1 h post-injection of CD34$^+$ cells in human patients with MI (Musialek *et al.*, 2011). Another study demonstrated that a majority of cultured early EPCs injected directly into the myocardium post-MI disappeared within a week (Cho *et al.*, 2007). This low engraftment of injected cells is a major problem in order to enhance therapeutic effects of cell therapy because stable engraftment and survival of implanted cells should be a prerequisite for 'cell' therapy. One recent study suggested an association between durable engraftment of the transplanted EPCs and maintenance of functional improvement in experimental diabetic neuropathy (Jeong *et al.*, 2009). When cultured, early EPCs were intramuscularly transplanted along the nerve, they engrafted into the diabetic nerve along the vasa nervorum for more than 12 weeks and improved neural function for more than 8 weeks (Jeong *et al.*, 2009).

Cells that successfully adhere to the extracellular matrix have a higher chance of survival by avoiding anoikis (apoptosis caused by lack of adhesion to extracellular matrix). This adhesion capacity may play an important role in cell survival, particularly in the ischaemic environment. CD31 was originally discovered as an adhesion molecule between cells (Xie and Muller, 1993; Zocchi and Poggi, 1993), and it has been shown to mediate cell–cell adhesion mainly through homophilic interactions between CD31-expressing cells (Woodfin *et al.*, 2007). Genome-wide gene expression data showed a high expression of genes related to adhesion, transmembrane structure, chemokine production and reception, and extracellular matrix in hPB-CD31$^+$ cells (Kim *et al.*, 2010c). Cell adhesion assays further demonstrated that CD31$^+$ cells have a higher adhesion capacity to various extracellular matrix proteins such as collagen, laminin, vitronectin and fibronectins than CD31$^-$ cells (Kim *et al.*, 2010a,c). In fact, the confocal microscopic data and FACS analysis for digested tissues verified higher engraftment of mBM-CD31$^+$ cells compared with mBM-CD31$^-$ cells in cell transplantation studies with a hindlimb ischaemia model. CXCR4/ SDF-1 is a well-known signalling axis to mediate cell engraftment and migration. However, the expression of CXCR4 was not different between CD31$^+$ cells and CD31$^-$ cells, suggesting that the higher engraftment of CD31$^+$ cells does not depend on this pathway. On the other hand, the CD31 molecule itself and other adhesion molecules ICAM4 and integrin α which were more highly expressed in CD31$^+$ cells than CD31$^-$ cells could have contributed to the higher engraftment of CD31$^+$ cells. The higher engraftment of CD31$^+$ cells is likely to augment the angiogenic and vasculogenic ability of mBM- and hPB-CD31$^+$ cells (Kim *et al.*, 2010a,c). CD31 is the first marker used to isolate a BM cell subpopulation that has higher adhesion and engraftment potential.

Together, BM-derived CD31$^+$ cells exert therapeutic effects mainly through non-transdifferentiation humoral effects. By avoiding cumbersome BM biopsy or cell mobilization used for isolation of other progenitor cells such as CD34$^+$ or CD133$^+$ cells, CD31$^+$ cell therapy will become a promising option for treating patients with advanced ischaemic cardiovascular diseases.

Enriched HSCs and HPCs

Gene expression studies have shown that the levels of haematopoietic stem and progenitor cell genes were higher in mBM-CD31$^+$ cells and hBM-CD31$^+$ cells than mBM-CD31$^-$ cells and hBM-CD31$^-$ cells respectively (Kim *et al.*, 2010a), supporting the notion that haematopoietic stem and progenitor cells are enriched in the CD31$^+$ cells. FACS analysis confirmed that more than 90% of HSCs, multipotent progenitor cells, common lymphoid progenitor cells and common myeloid progenitor cells in mBM express CD31 (Kim *et al.*, 2010a). *In vitro* colony-forming assays and *in vivo* BM cell transplantation experiments further support that haematopoietic stem and progenitor cells are almost exclusively included in mBM-CD31$^+$ cells (Kim *et al.*, 2010a). Similarly, in hBM, CD31 was expressed in 99.8% of CD34$^+$CD133$^+$ and 89% of CD34$^+$CD133$^-$, indicating that HSCs and most HPCs express CD31 (Kim *et al.*, 2010a). *In vitro* haematopoietic colony-forming assays revealed that clonogenic HPCs are enriched in hPB-CD31$^+$ cells (Kim *et al.*, 2010c). These studies indicate that HSCs and HPCs are almost exclusively included in the CD31$^+$ cell fraction.

CD34$^+$ cells are effective for improving ischaemic cardiovascular diseases in animal models (Kawamoto *et al.*, 2003) and human patients (Losordo *et al.*, 2007; Kawamoto *et al.*, 2009). Given that HSCs/EPCs that are heavily enriched in the CD34$^+$ cell fraction are almost exclusively present in the CD31$^+$ population, and that HSCs/EPCs have been reported to be instrumental for therapeutic neovascularization in ischaemic cardiovascular diseases, it is questionable whether HSC populations included in CD31$^+$ cells are responsible for ischaemic cardiovascular repair. When this population (hBM-CD34$^-$CD31$^+$ cells) was compared with hBM-CD34$^+$ cells, therapeutic effects between these two groups were similar in improving mouse limb ischaemia, suggesting that non-HSC populations among the CD31$^+$ cell fraction play important roles in therapeutic neovascularization.

Clinical application

One major advantage of CD31$^+$ cells over CD34$^+$ or CD133$^+$ cells is their prevalence in circulating blood. Approximately 30–35% of total MNCs of hPB are CD31$^+$. For instance, if 60 million CD31$^+$ cells are needed for transplantation, only 100 mL of blood is needed (Kang *et al.*, 2004; Kawamoto *et al.*, 2009). Thus, there is no need to use mobilizing agents, such as G-CSF, for collecting CD31$^+$ cells (Kawamoto *et al.*, 2009; Losordo *et al.*, 2011). This leads to the reduction in cost of cell therapy, simplification of treatment procedures and removal of potential adverse effects associated with mobilizing agents. Another advantage of CD31$^+$ cells over the cultured EPCs or MSCs is the avoidance of cell culture. The major drawback of cultured cells for clinical use is culture-associated side effects. Current EPC or MSC expansion protocols utilize media containing fetal bovine serum. This use of xenogeneic serum can pose a risk such as disease transmission

through viral, prion, and zoonose contamination or immunizing effects. One early study reported that patients receiving repeated MSC transplantation developed anti-fetal bovine serum antibodies and elicited inflammatory and immunological reactions, provoking influencing the therapeutic results (Horwitz *et al.*, 2002). Another potential benefit over uncultured BM-derived MNCs or unfractionated cells is the removal of unnecessary cells that may induce adverse effects such as calcification (Yoon *et al.*, 2004) or aggravation of ischaemia (Miyamoto *et al.*, 2006). In the studies using $CD31^+$ cells, no adverse effects were observed (Kim *et al.*, 2010a,c). Recent studies suggested that BM-MNCs (Iso *et al.*, 2010) or G-CSF-mobilized PB-MNCs (Horie *et al.*, 2010) are effective in the treatment of patients with critical limb ischaemia. When we compared hBM-MNCs with hBM-$CD31^+$ cells in a mouse model of hindlimb ischaemia, the therapeutic efficacy of $CD31^+$ cells was superior to that of BM-MNCs. Thus, $CD31^+$ cell selection by removing nonangiogenic and highly inflammatory cells included in the $CD31^-$ cell fraction appears to have higher therapeutic effects and less potential toxicity.

Future perspective

One barrier to the use of EPCs, particularly the uncultured EPCs in cardiovascular regeneration, is the low number of cells to be obtained from the PB. In addition, the number and/or function of EPCs are reported to be reduced in patients with advanced age (Heiss *et al.*, 2005), diabetes (Ii *et al.*, 2006), hypercholesterolaemia (Vasa *et al.*, 2001) or hypertension (Vasa *et al.*, 2001; Imanishi *et al.*, 2005), and in patients who smoke (Kondo *et al.*, 2004; Michaud *et al.*, 2006). Thus, the clinical success of EPC therapy may depend on the development of efficient methods to increase the number and/or potency of EPCs.

To identify more angiogenic and/or vasculogenic cells, further experimental investigation is required. As $CD31^+$ cells are a heterogeneous cell population that includes T and B lymphocytes and myelomonocytic cells, more restricted angiogenic or vasculogenic cells can be identified by using additional markers. The addition of other markers will help narrow down the identification of more specialized cells to possess higher angiogenic and vasculogenic potency. Mechanistically, the role of CD31 itself in mediating neovascularization in the ischaemic tissues needs to be determined. Although CD31 knockout mice display a grossly normal phenotype (Thompson *et al.*, 2001), studies with tissue- and time-specific conditional deletion or overexpression of CD31 genes are required to elucidate this role.

As experimental proof has been provided, clinical trials with $CD31^+$ cells should follow. The first target could be the patients with critical limb ischaemia or non-healing wounds. There are a number of reports that the efficacy of myocardial repair or regeneration with BM-derived cells may be minimal or modest. However, studies with unselected BM or PB-MNCs for treating peripheral vascular obstructive disease has shown to be effective (Al Mheid and Quyyumi, 2008). In addition, a recent presentation showed that $CD34^+$ cells are effective in the treatment of critical limb ischaemia patients (Losordo *et al.*, 2010). Peripheral tissues have more room for cell engraftment and present a less hostile environment for cell survival. As such, critical limb ischaemia could be a good initial target for cell therapy with $CD31^+$ cells. A recent diabetic neuropathy study also indicated that the host environment is a more important factor in determining engraftment and survival (Jeong *et al.*, 2009; Kim *et al.*, 2009). Upon success with critical ischaemia, this cell therapy can be expanded to the treatment of other ischaemic cardiovascular diseases including MI, stroke and diabetic neuropathy.

A decade of experience with BM cell therapy for cardiovascular diseases has yielded novel insight into the underlying mechanisms. Important discoveries include the major role of the humoral and paracrine mechanisms, the importance of the host environment in cell engraftment and therapeutic effects, and the advantages of selected cells. Based on these new findings, we believe that now is the time to rethink the therapeutic usefulness of currently used cell types. As far as therapeutic effects are not heavily dependent upon tissue generation from transplanted cells, we may not need to adhere to using stem or progenitor cells which only exist in low number and requires special measures for isolation, but to focus on identifying and applying specialized angiogenic and/or vasculogenic effector cells for therapy. Furthermore, tailored therapy according to the type of target disease should be considered. Additionally, engineering of cells with biomaterials is required to improve cell function, delivery and retention.

Acknowledgements

This work was supported in part by NIH grants DP3DK094346, RC1GM092035; and NIH contract, HHSN268201000043C (Program of Excellence in Nanotechnology Award); Wallace H. Coulter Translational Research Grant; Pilot grant of Emory-Georgia Tech Regenerative Medicine; NSF-EBICS (Emergent Behaviors of Integrated Cellular Systems) grant, CBET-0939511; and Stem Cell Research Center of the 21st Century Frontier Research Program grant SC4300, funded by the Ministry of Science and Technology, Republic of Korea.

Conflict of interest

The authors indicate no potential conflict of interest.

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