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Mesenchymal Stromal Cells in Cardiovascular Disease

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Abstract

Mesenchymal stem cells (MSCs) can be isolated from different adult tissues and can be differentiated along stromal lineages (i.e. osteoblasts, adipocytes, and chondrocytes). Recent data in the literature provided evidence that MSCs can also be differentiated *in vitro* into additional cell types, such as endothelial cells. In our studies we showed the endothelial differentiation and angiogenic potential of human neonatal foreskin derived stromal cells (hNSSCs) both *in vitro* and *in vivo*.

In vivo experiments demonstrated significant therapeutic capabilities of infused MSCs in various cardiovascular disease models. Notably, the majority of studies reported minimal trafficking and/or persistence of infused cells at the site of injury, suggesting secretion of paracrine factors as a more reasonable mechanism by which MSCs repair damaged tissues. In light of these findings, several human clinical trials utilizing MSCs for cardiovascular diseases were initiated. Published data from these trials substantiated significant therapeutic benefits of transplanted MSCs. However, remaining challenges in the field are at least four fold: i) how to isolate MSCs in higher purity, ii) how to grow them in sufficient numbers for *in vivo* applications, iii) how to direct them to the site of injury and, iv) how to improve their therapeutic properties. In this chapter we review published literature documenting the endothelial differentiation capability of MSCs and will discuss the pre-clinical as well as the clinical utilization of MSCs for cardiovascular diseases.

Keywords

Mesenchymal Stromal Cells, Endothelial Cells, Cardiovascular, Clinical Trial

Introduction

The characterization of mesenchymal stemcells (MSCs) relies mainly on the study of *in vitro* culture-developed cell populations. Despite years of intense research, the position and function of resident MSCs within their origin *in vivo* are unknown, due to lack of specific markers to identify naive MSCs [1]. The probability exists that the MSC surface protein expression may fluctuate between *in vivo* and *in vitro* settings, because of the loss of MSCs from their niche and the use of physical and chemical growth environments. Phenotypic characteristics of MSCs are not stable during *ex vivo* treatment; as MSC's can undergo some changes including loss of specific markers and acquisition of new markers [2]. To resolve this issue, the International Society for Cell Therapy (ISCT) proposed in 2006 the following minimal criteria for the minimal definition of human MSCs: i) adherence to plastic in standard culture conditions; ii) must express stromal cell associated markers (such as $CD73^+$, $CD90^+$, $CD105^+$) and must not express hematopoietic and endothelial markers (such as $CD34^-$, $CD45^-$, HLA-DR^-, $CD14^-$ or $CD11b^-$, $CD79a^-$ or $CD19^-$) as assessed by flow cytometry analysis; and iii) *in vitro* differentiation capability into adipocytes, osteoblasts and chondroblasts *in vitro* [3]. MSCs have been successfully isolated from several species, including humans and mice. While MSCs are routinely isolated from bone marrow (BM), it is now evident that MSCs can also be isolated from many other tissues, including peripheral blood, cord blood, cord wharton's jelly, adipose tissue, amniotic fluid, compact bone, periosteum, synovial membrane and synovial fluid, articular cartilage and foetal tissues. Although MSCs isolated from different sources share similar surface antigens, and exhibit similar classical differentiation potential (bone, fat, and cartilage), these cells still exhibit heterogeneity in their phenotype and biological properties, which apparently depends on their tissue of origin and microenvironmental niche [4]. Therefore, in contemporary regenerative medicine, we can split MSCs can be divided according to their source into two broad categories, marrow derived and non-marrow derived. The isolation of MSCs focusing on non marrow-derived sources has been described, because the repeated isolation of bone marrow-derived MSCs can be compromised as a result of viral infection, the decline in the number of stem cells with age, or the need for a highly invasive procedure [5].

Endothelial cells are currently regarded as an integral part of tissue repair and regeneration. However, mature endothelial cells have inadequate proliferative capacity *in vitro* and *in vivo*; hence there is an urgent need to explore alternative sources of cells for autologous and allogenic transplantation applications. Recent progress has made it possible use stem cells as cell sources for therapeutic angiogenesis, as well as in the vascularization of engineered tissue grafts. This chapter will present data from the literature documenting successful utilization of MSCs for cardiovascular disease under pre-clinical and clinical settings.

Endothelial Differentiation of MSCs (*in vitro*)

One of the criteria utilized to define MSCs is the capability to differentiate *in vitro* into the three classical stromal lineages, namely fat, bone and cartilage. Recently, the spectrum of their differentiation potential was extended to angiogenesis. MSC sources may also hold progenitor cells with angiogenic capability (i.e. to differentiate into visceral mesoderm origin such as endothelial cells and to contribute

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to blood vessel construction). Identification of the ideal therapeutic populations remains an essential aspect in regenerative medicine. *In vitro* aids this aim by selecting populations that are appropriate for more exclusive (but time-consuming) *in vivo* evaluation. Although *in vitro* methods cannot completely mimic the repertoire of cellular and molecular actions that occur *in vivo*. Nonetheless, it is feasible to develop approaches that imitate essential basics of the *in vivo* systems. *De novo* blood vessel formation (neovascularization) is essential for the revitalization of ischemic tissue in adults. Formerly, it was assumed that the mechanism for developing new vascular arrangements in the context of growth or tissue ischemia is angiogenesis (i.e., the sprouting of microvessels from a preexisting capillary bed) [6]. However, recent data suggests that adult bone marrow derived cells can be involved in angiogenesis through the recruitment of endothelial progenitor cells in response to signals produced by damaged tissues (postnatal vasculogenesis) [7]. Vasculogenesis-mediated blood vessel expansion is altered in adults in numerous pathological conditions, such as peripheral vascular disease, myocardial ischemia and infarction, stroke, wound healing, retinopathy and tumor growth [8]. Endothelial progenitor cells hold great promise as potential cell-based therapies for such disease conditions [7]. Subsequent studies have shown that non-marrow derived sources like peripheral blood, umbilical cord blood, cord tissue and adipose tissue derived cell population also have neovascularization property *in vitro* and *in vivo* [9-14]. Proliferation and survival of endothelial cells is an essential aspect of angiogenesis whereas adipose stromal cells (ASCs) have the capability to support endothelial cell growth by secretion of proangiogenic growth factors, particularly VEGF (vascular endothelial growth factor) and HGF (hepatocyte growth factor). This event has been investigated in hypoxic (1% CO_2) and normoxic (1% CO_2) conditions, with ASCs typically displaying a higher response in hypoxic condition [15]. Other than MSCs, progenitors for endothelial cells have been found both in bone marrow and peripheral blood. Since bone marrow-derived circulating endothelial progenitor cells (EPC) are involved in postnatal neovascularization, this has suggested that utilization of a cell-based therapeutic angiogenesis might be possible [16]. Peripheral blood EPCs and bone marrow derived multipotent adult progenitor cells (MAPCs) were found to be positive for vascular endothelial growth factor (VEGF) receptor 2, also known to as FLK1 or KDR. EPCs were positive for CD34 and CD133, and MAPCs were dimly positive for CD44 and CD133 [17-19]. EPCs can be mobilized from bone marrow either exogenously by cytokine stimulation (i.e. HMG-CoA reductase inhibitors) or endogenously by tissue ischemia [20,21]. However, clinical application of EPC is a cumbersome procedure due to their scarcity, essentially in patients who could benefit most from such therapeutic angiogenesis [21]. Therefore, MSCs from different sources have recently been investigated for their angiogenic potential, aside from their well-established mesoderm differentiation properties.

Several studies have portrayed the classical multilineage differentiation of MSC to adipocyte, osteoblast and chondrocyte *in vitro* by appropriate induction factors [22]. Similarly, to promote endothelial differentiation, MSC cultures are incubated in low-serum conditions supplemented with different factors such as VEGF (Vascular endothelial growth factor), basic fibroblast growth factor (bFGF/ FGF2), ascorbic acid (vitamin C), epidermal growth factor (EGF), heparin, hydrocortisone and Insulin-like growth factor 1 (IGF-I) [11,22-24]. These agents converted MSCs into endothelial-like cells without altering their morphology when continuously exposed for 7 days. The resulting differentiated MSCs displayed several features characteristic of endothelial cells, including the expression of a number of endothelial-associated genes. *In vitro* endothelial differentiation is usually assessed by several assays like immunofluorescence for PECAM (CD31), von Willebrand factor (vWF) in Weibel-Palade bodies, CD34, VEGF receptors (KDR and FLT-1), VE-cadherin (CD144), eNOS and VCAM-1(CD106). Other assays are matrigel tube formation, Ac-Low-density lipoprotein (LDL) uptake, and 6-keto prostacyclin secretion assay (radioimmunoassay) [11,23,24]. When cultured with endothelial growth factors, bone marrow MSCs displayed a strong expression of endothelial-specific markers such as VEGF receptors (VEGFR-1 and VEGFR-2) and vWF [23]. It is noteworthy that the endothelial differentiation process was found to be reversible because switching MSCs back to a routine culture environment led to the disappearance of endothelial characteristics, while upon returning to endothelial environment, the endothelial features reappeared [24]. Therefore it might be justified to speculate that under the aforementioned culture conditions, the differentiated cells may have not reached a terminally differentiated endothelial state, i.e. the differentiated cells may not have left the mesenchymal stem cell compartment. The essential role for vitamin C was well described in a human aortic endothelial cells (HAEC) study, whereas HAEC cultured in the absence of vitamin C were basically scorbutic. However, supplementation with vitamin C reduced the oxidative stress significantly and increased the level of GSH, GSH/GSSG ratio and eNOS activity in human aortic endothelial cell culture [25]. Similarly, oxidative stress, low LDL-uptake and morphology changes were noticed in human MSC endothelial differentiation cultures lacking vitamin C [24]. The omission of other inducible factors such as EGF, IGF and VEGF from endothelial differentiation culture negligible effect on MSC's LDL-uptake, expression of endothelial markers and endothelial tube formation. In contrast, the exclusion of b-FGF significantly impaired MSC's LDL-uptake [24].

The matrigel system has been used broadly for *in vitro* angiogenic assays, because it provides the necessary extracellular matrix molecules and essential growth factors [26]. Matrigel is a soluble and sterile extract of basement membrane proteins derived from the EHS (Breth-Holm-Swarm) tumor that forms a 3D gel at 37°C and supports cell morphogenesis, differentiation, and tumor growth. Mesenchymal cells such as MSCs, pericytes, or fibroblasts co-align when cultured on matrigel [27]. Matrigel promotes the differentiation of various different cell types as well as the outgrowth of differentiated cells; however, it does not induce the proliferation of such cells. Both the morphology and the gene expression profile of cells grown on matrigel revealed a more differentiated phenotype [28]. Cells on or in this matrix associate with each other, usually in three dimensions, and then generate structures similar to those formed at their origins. Endothelial cells begin to attach to each other and align within an hour and form capillary-like structures with a lumen within 24 hours [29]. Primary and immortalized microvascular endothelial cells and human umbilical vein endothelial cells (HUVEC) form nearly identical capillary-like structures on this substratum [28-30]. Similarly, induced MSCs were shown to form a vascular network when cultured with endothelial medium on matrigel. Morphological changes were observed at different time points, during the first 12h, cells spread randomly and started to form seldom interconnected clusters, followed by an increase in the cluster size with highly connected capillary tube-like structures after 12h and 48h, and finally discrete matrigel areas were empty and surrounded by cell islets [12,13,23]. Our *in vitro* and in vivo studies also confirmed the endothelial and angiogenic property of the hNSSCs by utilising the above-mentioned investigations [31,32].

Utilization of MSCs for Cardiovascular Diseases (Pre-clinical)

Despite recent progresses in the clinical management of cardiovascular disorders, this group of diseases remains the leading cause of death worldwide, underscoring the need for exploring novel therapeutic strategies. Such novel therapeutic modalities could be based on MSCs as they have recently emerged not only as capable of immunoregulation and differentiation, but also of tissue repair incardiovascular disease. Since the early 2000s, several preclinical studies have shown promising efficacy of MSCs for the treatment of various cardiovascular diseases in animal models. Orlic and colleagues demonstrated that transplantation of Lin c-Kit$^+$ bone marrow (BM) cells could regenerate the infarcted myocardium in mice [33]. Subsequently, the same group demonstrated that transplantation of mobilized (by stem cell factor (SCF) and granulocyte-colony stimulating factor (GM-CSF)) blood mononuclear cells (BMC)

could regenerate the infarcted myocardium and prolong the survival of experimental mice [34]. Whereas these studies do not directly implicate MSCs in heart regeneration, Shake et al. demonstrated that MSCs can indeed home to and improve the function of infarcted myocardium [35]. Later on, a number of other studies showed that injection of MSCs could improve cardiac function through differentiation into endothelial cells and/or myocytes [36-40]. Similarly, Quevedo and colleagues reported that allogeneic MSCs can restore cardiac function in chronic ischemic cardiomyopathy through differentiation into cardiac, vascular muscle, and endothelial lineages [41]. While transplantation of MSCs lead to significant reduction in myocardial scar size in rats, Jaquet and colleagues observed no evidence of transdifferentiation or neovascularization in those animals [42]. Similarly, tracking experiments demonstrated that infused MSCs were not present at the scar site at four weeks post-injection, despite evident therapeutic benefit [43]. These and other findings led to the assumption that MSCs might repair damaged heart via other mechanisms rather than transdifferentiation. In concordance with this hypothesis, Gnecchi et al reported that functional recovery in the hearts of myocardial infarction (MI) rats transplanted with AKT-modified MSCs occurred within 72 hrs post implantation, suggesting alternative mechanisms by which MSCs might exert their effects [44]. The authors suggested a paracrine effect of MSCs wherein administration of AKT-MSC conditioned medium significantly minimized the infarct size and improved heart function, again suggesting a paracrine effect of MSCs (through secretion of VEGF, FGF-2, HGF, IGF-I, and TB4) as an alternative mechanism by which MSCs can remodel damaged heart and improve its function. Despite a number of published reports documenting successful utilization of MSCs to treat numerous cardiovascular disease models, one major problem was the low survival rate of transplanted MSCs in animals. In an attempt to overcome this limitation, Li et al utilized BCL-2-engineered MSCs and reported better survival of the transplanted MSCs, which was associated with better therapeutic benefit compared to normal MSCs [45]. In another study, Pons et al reported that co-injection of MSCs with VEGF led to pro-longed survival of MSCs and better outcome in MI animal model [46]. Similarly, Fan and colleagues reported that transplantation of survivin-modified MSCs in a rat model of MI led to better therapeutic outcome compared to MSCs alone, which was associated with increased VEGF expression, increased vascular density, and reduction in infarction size [47]. Krausgrill et al. utilized MSCs treated with PDGF-BB prior to intramyocardial injection into MI rats, which was associated with prolonged survival of transplanted cells in the infracted hearts [48]. As an alternative strategy, a number of other studies have looked at using genetically-modified MSCs to enhance vasculogenesis. Sun et al. utilized angiopoietin-1-engineered MSCs and reported an enhanced therapeutic efficacy of those cells, which was associated with increased angio- and arteriogenesis [49]. In another study, Zang et al. reported successful utilization of MSCs co-injected with erythropoietin in a rat model of myocardial infarction [50]. Deuse and colleagues successfully utilized *HGF* or *VEGF*-expressing MSCs in a mouse model of acute myocardial infarction (AMI) [51]. Tang et al. reported that MSCs genetically modified to express SDF-1 and VEGF were highly efficient in enhancing cardiac function after MI, associated with increased vascular density, thicker left ventricle, and improved cardiac function [52]. A number of other studies reported that injection of SDF-1-expressing MSCs or MSCs plus SDF-1 led to enhanced efficacy and an increase in vascular density, and in one study, was associated with endothelial differentiation of transplanted MSCs [53,54]. Aside from these studies, Du et al. have reported immune-modulation as an alternative mechanism by which MSCs could repair the damaged heart. Injected MSCs were found to inhibit NF-kB activity, reduce TNF-α and IL-6 production, and to increase the production of IL-10 in the myocardium, collectively leading to lower inflammation and better outcome [55]. In an attempt to enhance the homing of MSCs to the ischemic heart, Schenk et al reported that over-expression of MCP-3 in heart cells led to better recruitment of infused MSCs to the infarcted heart, which was associated with improved function [56]. In another study, Mias et al. reported that the improved heart function in a MI rat model was associated with enhanced MMP2/MMP9 secretion by cardiac fibroblasts [57]. Recently, it was shown that secreted frizzled-related protein 2 (sFRP2) prolonged the survival of infused MSCs through inhibition of both Wnt and bone morphogenic protein (BMP) signaling pathways, collectively leading to better therapeutic efficacy [58]. While most of the aforementioned work focused on the ability of MSCs to differentiate into vascular and cardiac cells or through a paracrine effect, Hatzistergos et al. reported that the effect of MSCs in MI is mainly through the expansion of endogenous c-kit(+) cardiac stem cells (CSCs) [59]. Nonetheless, Song et al. recently reported that Cardiomyocytes derived from phorbol myristate acetate-activated MSCs had the capacity to restore electromechanical function in the hearts of MI rats [60]. Therefore it would appear from these data that MSCs can exert their therapeutic function in cardiovascular disease animals through multiple mechanisms including differentiation, secretion of pro-angiogenic factors, mediating anti-inflammatory response, or by stimulating the expansion and differentiation of endogenous stem cells.

MSCs in Tissue Engineering for Cardiovascular Therapy

As the predominance and prevalence of vascular disease continues to increase, the need for a suitable arterial replacement has encouraged scientists to explore the field of tissue engineering. Hence the number of studies related to vascular *tissue engineering has* grown dramatically. For decades, tissue engineering approaches have been used to induce vessel formation through expression of angiogenic factors and revascularization. The cells responsible for new vessel formation are endothelial cells, which offer significant potential in cell therapy for vascular diseases and ischemic tissues, and in many tissue engineering applications such as vascular grafts and pre-vascularized tissue beds [61]. Due to the ability of MSCs to differentiate into numerous types of tissues including bone, cartilage, muscle, tendon, fat, endothelial tissue, and smooth muscle, there has been great interest in utilizing these cells to engineer a fully functional blood vessel for vascular applications [23,62]. In 1993, Galmiche *et al.* demonstrated that MSCs isolated from human peripheral blood can adhere to plastic-surface culture and express smooth muscle α-actin positive microfilaments as well as other smooth muscle-specific proteins such as metavinculin, hcaldesmon, smooth muscle myosin heavy chain, and calponin at 3 to 7 weeks of culture [63].

In 1986, Weinberg and Bell constructed the first vascular tissue engineered *in vitro* which represented a multilayered artery with the lumen lined by endothelial cells (to prevent thrombogenicity and intimal proliferation), while the rest of the vessel consisted of multiple layers of collagen integrated within a Dacron mesh [64]. Even though the mechanical properties of this engineered vessel were insufficient for *in vivo* use, this model proved to be a milestone in vascular surgery and science. In 1999, Niklason and colleagues developed a vascular graft material from smooth muscle and endothelial cells that were derived from a biopsy of vascular tissue produced *in vitro* under pulsatile conditions before implantation. These engineered vessels were then implanted in miniature swine animal models, where they remained completely patent for up to 2 weeks with patency documented by digital angiography [65]. This idea was then taken to the next level by Hoerstrup et al., who used an *in vitro* pulse duplicator system (bioreactor) to provide a "biomimetic" environment during tissue formation to yield more mature, implantable vascular grafts. In this system, a bioabsorbable polymer (polyglycolic-acid/poly-4-hydroxybutyrate) scaffold was seeded with bovine vascular myofibroblasts and endothelial cells and was then exposed to the static culture conditions of pulsatile flow to measure the burst pressure and the suture retention strength [66]. Such biomimetic system involves seeding bovine aortic smooth muscle cells into hollow tubular polyglycolic acid (PGA) scaffolds, followed by injection of bovine aortic endothelial cells into the lumen [67]. Nieponice and his group developed and *in vivo*-tested stem cell-based tissue engineered vascular graft for arterial applications by using poly (ester urethane) urea

compound scaffolds created by thermally induced phase separation (TIPS) with an outer electrospun layer of the same biomaterial (ES-TIPS). Some smooth muscle like layer of cells were observed near the luminal surface that stained positive for smooth muscle α–actin and calponin [68]. In 2008, Mettler and colleagues successfully created an autologous tissue-engineered pulmonary artery using progenitor cells co-seeded with stem cells, which was shown to be functional in vivo [69]. Stem cells are considered an invaluable cell source for regenerative medicine, given of their differentiation potential and proliferative capability. The innovative use of stem cells for vascular tissue engineering has opened new possibility for a fully engineered blood vessel. The endothelial progenitor cells (EPCs) were isolated from peripheral blood mononuclear cells and were shown to integrate into neovascularization and to differentiate into new endothelial cells and to generate hematopoietic stem cells [70,71]. Pre-clinical studies indicated that (EPCs) residue in the marrow and circulate in the blood at very low levels (<0.01% of all cells) [72]. Their prevalence in the blood can change in response to various stimuli. Ischemia increases VEGF expression, which in turn activates matrix metalloproteinases, which releases the EPCs ($CD34^+$/c-Kit^+ cells) from the vascular niche by cleaving Kit-ligand. Subsequently the mobilized EPCs enter the circulation and home to the site of ischemia [17,71,73]. Engineered tissues, theoretically, have the ability to grow and remodel, and have less chance for rejection, thrombosis, and infection compared with synthetic tissues. Rezai et al. has raised the therapeutic possibility of using bone marrow-derived stem cells as a source of cells for tissue repair and regeneration which provides a less invasive source of cells applicable in tissue engineering applications, including cardiovascular tissues such as heart valves, blood vessels, and myocardium [74]. In 2010, Phelps and colleagues engineered polyethylene glycol-based bioartificial hydrogel matrices presenting protease-degradable sites and cell-adhesion motifs. To induce the growth of vasculature *in vivo*, the authors delivered sustained *in vivo* levels of VEGF over 2 weeks as the matrix degraded. When implanted subcutaneously in rats, VEGF induced a significant number of vessels to grow, as assessed by increasing vessel density at 4 weeks post-implantation [75]. Dargaville et al. reported the utilization of a series of copolymers of trimethylene carbonate (TMC) and l-lactide (LLA) as scaffold to grow human MSCs. Interestingly, when these scaffolds were implanted into the rat peritoneal cavity, it stimulated the formation of tissue capsules, containing myofibroblasts [76]. Recently, Rustad et al. reported successful utilization of a biomimetic pullulan-collagen hydrogel scaffold in an excisonal wound healing model, which enhanced the angiogenic capability of bone marrow-derived murine MSCs. It is noteworthy that growing MSCs on this scaffold led to enhanced angiogenicity of the MSCs, which was associated with the secretion of several angiogenic factors and the transcription of genes associated with pluripotency [77]. Similarly, Godier-Furnémont et al reported successful growth of human mesenchymal progenitor cells (MPCs) on a cell-matrix composite scaffold. When implanted onto the infarct bed in a nude rat model of cardiac infarction, MPCs greatly enhanced vascular formation in the infarct bed through the secretion of paracrine factors, including SDF-1, and the migration of MPCs into ischemic myocardium [78].Nanofabrication techniques are currently under way to engineer artificial network structures that will mimic the capillary network expanding from a main vessel and merging back to a single vessel-like vein, with the ultimate goal of being utilized in tissue engineering applications [79].

Apart from the differentiation potential, MSCs has anti-inflammatory properties with paracrine actions. Previous works has revealed that MSCs release extracellular vesicles (EVs) differently depending on external motivation proposing that this process are regulated by cross talk between MSCs and their microenvironment [80,81]. EVs are able to affect cell characteristics such as phenotype, enrolment, proliferation, and differentiation in a paracrine action. EVs are mainly released from the endosomal compartment and their paracrine effects have a potential advantage in regenerative medicine particularly in cell viability, immune responses, ECM interaction, senescence and angiogenesis. EVc has been combined in various regenerative therapies, for example mixing with hydrogels, co-injection, coating scaffolds with specific linkers [82]. The therapeutic application of MSCs derived EVs have been studied in various models such as various heart conditions, liver injury, kidney injury, lung injury and wound healing [83]. Particularly, the conditioned media obtained from hMSCs have the potential of cardio protective effects

Table 1: Clinical Trials of Stem Cells in Cardio-Vascular Diseases

Sample Size	Cell Type	Study Design	Delivery Route	Outcome	Ref
Acute Myocardial Infarction/Heart Failure					
0	Autologous mononuclear bone marrow cells	Catheter placed into the infarct-related artery	Intracoronary transplantation	myocardial regeneration and neovascularisation	[87]
69	Autologous bone marrow mesenchymal stem cell		Intracoronary injection	Improvement in left ventricular function	[88]
18	Autologous mononuclear bone marrow cells		Intracoronary transplantation	Functional and metabolic regeneration of infracted and chronically vital tissue	[89]
25	BM-MNC		Intramyocardial Injection	Sustained beneficial effect on anginal symptoms, myocardial perfusion, and left ventricular function	[90]
46	Autologous of BMCs		Intracoronary transplantation	Improves heart rate variability	[91]
15	Autologous Bone Marrow mononuclear cells		Injection into the infarction border zone	Decrease in heart failure symptoms and an improved left ventricular (LV) function.	[93]

Sample Size	Cell Type	Study Design	Delivery Route	Outcome	Ref
Acute Myocardial Infarction/Heart Failure					
5	Autologous bone marrow derived MSC		Intracoronary transplantation	Slight improvement in myocardial function in 3 patients	[92]
32	Administration of BMCs		Intracoronary infusion	No change in LV ejection fraction could be demonstrated after repeated	[104]
30	Autologous bone marrow cells		Direct intramyocardial injection	Improvement in symptoms achieved in approximately 50% of patients	[94]
30	Autologous mesenchymal SCs (MSCs) and (BMCs)	Randomized, double-blind	Intramyocardial Injection	Safety and efficacy (determined primarily by cardiac magnetic resonance imaging)	[105]
53	Bone marrow-derived allogeneic hMSCs	Randomized, double-blind, placebo-controlled, dose-escalation study	Intravenous injection	Intravenous allogeneic hMSCs are safe in patients after acute MI	[106]
20	Autologous bone marrow mononuclear cell (ABMMNC)	randomized study	transendocardial injection	ABMMNC therapy is safe and improves symptoms, quality of life in patients with chronic HF	[107]
17	Bone marrow stem cells (BMSCs) have been used to treat	Randomized	Intracoronary route	The left ventricular end-systolic volume (LVESV) and wall motion score index (WMSI),left ventricular end-diastolic volume (LVEDV), LVESV, and WMSI were significantly reduced in BMSC group	[108]

Sample Size	Cell Type	Study Design	Delivery Route	Outcome	Ref
Peripheral arterial disease					
45	Bone marrow-mononuclear cells		Injection into the gastrocnemius of the ischaemic limb	Significantly improved in legs	[97]
7	Autologous bone-marrow mononuclear cell		Transplantation	Improvement in endothelial dysfunction	[98]
28	G-CSF with mobilized PBMNCs	Randomized study	Subcutaneous injections of diabetic patients	Lower limb pain and ulcers were significantly improved	[109]
1	Bone marrow mononuclear cells		Intramuscular and intraarterial injection	Improvement chronic limb ischemia attributed to increased neo angiogenesis	[110]
7	Autologous bone-marrow mononuclear cell		Transplantation	Improvement in endothelial dysfunction	[98]
28	G-CSF with mobilized PBMNCs	Randomized study	Subcutaneous injections of diabetic patients	Lower limb pain and ulcers were significantly improved	[109]
1	Bone marrow mononuclear cells		Intramuscular, intraarterial injection	Improvement chronic limb ischemia attributed to increased neo angiogenesis	[110]

Sample Size	Cell Type	Study Design	Delivery Route	Outcome	Ref
Peripheral arterial disease					
12	Autologous bone-marrow mononuclear cell		Transplantation	Significant improvements in rest pain and pain-free walking time	[99]
140	BMCs with mobilized PBMNCs	Randomized study	Transplantation in the ischemic necrosis	Significant improvement, reduction in edema and increased colaateral vessels formation	[111]
32	Autologous bone-marrow mononuclear cell		Intraarterial and intramuscular injection	Significant improvement in the lower limb ischemia	[112]
184	Bone marrow mononuclear cells		local injections	Significantly enhanced endothelial colony-forming cell adhesion	[113]
40	Autologous (BM-MNC)	Randomized study	Injection	Accelerates wound healing	[101]
527	Autologous (BM-MNC)	Randomized study	Injection	Significant improvement in the amputation rate, ulcer healing,	[114]

Sample Size	Cell Type	Study Design	Delivery Route	Outcome	Ref
Patient with Breast cancer					
30	HGigh dose- (HDCT) with (PBPCT)			Immune function improved with a statistically significant increase of lymphocyte count	[105]
	Bone-marrow derived stem and progenitor cells	Randomized, double-blind studies		Improvement of ankle-brachial index (ABI), reduction of pain, and decreased need for amputation	[102]

Abbreviations: BMCs: Mononuclear bone marrow cells, G-CS: Granulocyte colony–stimulating factor, PBMNCs: Peripheral blood mononuclear cells, BM-MNC: Bone marrow-derived mononuclear cells, HDCT: Chemotherapy, PBPCT: Peripheral progenitor cell transplantation

to reduce myocardial infarct size by 60% in a porcine model of cardiac ischemia/reperfusion (IR) injury [84]. Moreover, it has been studied in mouse model and confirmed the active, cardio-protective component of MSC-derived CM is, in fact, the EVs [85]. In an *in vitro* angiogenesis assay, Isolated EVs from MSCs cultured under hypoxic conditions, MSC-EVs had promoted HUVEC cell migration and tube formation that was comparable to that induced by VEGF. Furthermore, *In vivo* studies confirmed that hypoxia-conditioned MSC-EVs significantly improved cardiac function with similar effectiveness to that observed in a control group (whole-cell MSC) [86].

Clinical Application of Human Mesenchymal Stem Cells (Hmsc) in Cardiovascular Disease

Pre-clinical data has demonstrated successful utilization of MSCs to treat a number of cardiovascular diseases in various pre-clinical animal models. There are currently more than 40 registered clinical trials using human MSCs in patients with cardiovascular diseases (www.clinicaltrials.gov). Table 1 provides, at the time of working, an up-to-date list of published literature on the clinical trials utilizing hMSCs for cardiovascular diseases, several of which will be discussed in the following section.

Intracoronary Injection of Bone Marrow Stem Cells

In Acute Myocardial Infarction (AMI)

In a groundbreaking clinical trial in 2002, Strauer et al. demonstrated marked improvement in the left ventricular function of 10 patients infused with autologous mononuclear bone marrow cells (BMCs) 5–9 days following MI, compared to that in the control group receiving standard care [87]. After standard therapy for AMI, the bone marrow from 10 patients was aspirated from the ilium, and BMCs purified using ficoll density gradient. The BMCs were transplanted through a balloon catheter in the infarct-related artery during balloon dilation. A control group of 10 AMI patients was treated with standard therapy alone. The authors found at three months post-infusion that, the infarct region in the cell transplant group had significantly decreased and was significantly smaller than that in the control group. Wall movement velocity over the infarct region increased significantly only in the transplant group. Further examinations of the transplant group resulted in marked improvement in stroke volume index, left ventricular end-systolic volume and contractility, and myocardial perfusion. Hence, this study demonstrated that transplantation of autologous BMCs was both safe and effective in reducing the impact of MI through myocardial regeneration and neovascularization. In another bone marrow mesenchymal stem cell (BMSC) trial for AMI reported by Chen et al. [88], 69 patients received angioplasty, followed by stent deployment within 8 ± 3.7 hours of MI. Eight days after the initial intervention, autologous bone marrow was aspirated from the ilium of each patient and cultured for 10 days to expand the cells. The BMSCs were transplanted through an inflated balloon catheter into 34 patients, while the control group (35 patients) received saline. All patients were measured for myocardial viability and cardiac function on the day of transplant, and follow up tests were performed at 3 and 6 months post-transplant. Fifteen patients in the BMSC group and eight in the saline group were interrogated by electromechanical mapping (EMM) one day prior to and three months post transplantation. The study found a significant decrease in the percentages of hypokinetic, akinetic, and dyskinetic segments of the left ventricle in the transplant group. Wall movement velocity over the infarct region increased significantly after transplantation. The left ventricular function fraction increased significantly in the BMSC-treated group at the 3-month mark with no change at 6 months. Perfusion defects decreased significantly for transplantation group at 3 months as demonstrated by positron emission tomography, while end-diastolic

volume decreased significantly as well. EMM results showed that the BMSCs infiltrated quickly into the infarct zone and remodeled the left ventricle. In addition, the study demonstrated that the transplantation of BMSC could survive well beyond the 7–14 day window previously postulated by Strauer and colleagues [87].

In Chronic Myocardial Infarction (CMI)

In 2005, Strauer and colleagues published their ongoing study providing intracoronary autologous mononuclear BMCs transplants in patients with CMI that led to marked metabolic and functional improvements [89]. The Strauer team treated 18 consecutive patients with CMI (for 5 months to 8.5 years) with BMCs, and compared them to a representative untreated control group. A 3-month follow-up showed significant reduction in infarct size. Both left ventricular ejection and wall movement velocity were significantly increased, while no marked changes were seen in the control group. The authors reported an increase in oxygen and glucose uptake in the infarct tissue, demonstrating the beneficial therapeutic effect of BMC therapy on cardiac performance in CMI.

In refractory angina

Other clinical studies have examined other symptoms associated with CMI. Beeres et al. reported in 2006 that myocardial injection of BMCs into patients with refractory angina pectoris and CMI significantly reduced angina, and increased myocardial perfusion and left ventricular function [90]. In this study, bone marrow was aspirated from the ilium of 25 refractory angina patients and BMCs were isolated using ficoll density gradient. BMCs were then injected by balloon catheter into areas of the myocardium with stress-induced ischemia. Gated single-photon emission computed tomography was administered at baseline, 3, and 12 months for left ventricular function and myocardial perfusion evaluations. One death, which was due to intracranial hemorrhage and appeared to be unrelated to BMC transplant, was reported in the study. At 3, 6, and 12 months angina symptoms and quality of life were compared to baseline values, and the results showed a significant decrease ($P < 0.01$) in daily angina episodes and the use of sublingual nitrates. The transplant group also demonstrated a significant increase in myocardial perfusion and left ventricular function.

In heart rate modulation

In 2007, Schueller et al. reported that the transplant of autologous BMCs after MI significantly improved autonomic heart rate modulation over the 12-month follow-up [91]. In this study, 23 patients post-MI were treated with intracoronary BMCs and the 23 patients in the control group received standard care. Each patient was evaluated by Holter monitoring for 24 hours at baseline, 3 months, and 12 months. The study found that heart rate variability (HRV) measures increased significantly compared to the control group, which was unchanged. The increase in HRV measures correlated to a decrease in arrhythmia and sudden death. A second study in 2007 by Katritsis et al. addressed the concern that BMSCs transplant could increase arrhythmia. Katritsis and colleagues aspirated bone marrow from 5 patients who, due to a prior anteroseptal MI, were implanted with a cardioverter-defibulator (ICD) to control ventricular arrhythmias [92]. Mononuclear cells were isolated on ficoll density gradient and the cells were expanded for one week in culture before intracoronary transplantation into the descending coronary artery. Stress echocardiography evidenced myocardial repair. Although each patient experienced arrhythmias prior to transplantation, none showed any signs of ventricular arrhythmias post-transplant. While the study was small, the data indicated that treatment with BMSCs and endothelial progenitors do not appear to be pro-arrhythmic.

Intramyocardial injection of bone marrow stem cells in severe chronic myocardial infarction

New imaging and catheter technologies are moving studies towards direct implantation into the myocardium. In a small pilot study of 15 patients with CMI and severe ventricular dysfunction in 2007, Beeres and colleagues investigated the safety, feasibility, and efficacy of direct injection of BMCs into the myocardium [93]. Bone marrow was aspirated from the iliac crest on the morning of injection. The team used the NOGA® cardiac navigation system to electro-mechanically map the ischemic areas of the left ventricle. After mapping, a NOGA® catheter was used to inject BMCs into the viable myocardium bordering ischemic areas. The study results demonstrated that even in a severely cardio-compromised population direct implantation of BMCs using the NOGA® system, was well-tolerated and showed a positive outcome. At two and half months post treatment, one patient died of heart failure, which was not unexpected in a population with such advanced disease. At three months post treatment, the authors found a decrease in heart failure symptoms and improved left ventricular function. There was also significant wall thickness and myocardial perfusion improvements in the injected segments that did not change in the non-injected myocardial segments. Preliminary results published in 2011 from a study by Godino et al. reported that direct intramyocardial injection of autologous BMSC into patients with CMI resulted in improved symptoms in approximately 50% of patients in the first 6 months [94]. There was a corresponding improvement in quantitative scintigraphic stress test imaging as well. The therapy appeared to be safe and well tolerated; however, the study needs to be completed before final conclusions can be made.

MSCs in the Treatment of Peripheral Arterial Disease (PAD)

Intramuscular injection

Murohara and colleagues [95] and Kalka & colleagues [96] demonstrated in animal studies that human mononuclear cells from peripheral blood or cord blood increased the number of capillaries in hindlimbs in animal studies, which supported the premise that bone-marrow-mononuclear cell implantation into ischemic limbs could promote angiogenesis. In a pilot study, Tateishi-Yuyama and colleagues recruited 25 patients with unilateral critical limb ischemia [97]. Bone marrow was aspirated from the ilium, and mononuclear cells were separated using a blood-cell separator. The BMSCs were injected into the gastrocnemius muscle of the ischemic leg, and saline was injected into the healthy limb. A second group of 22 patients with bilateral ischemia were recruited in the same study. Twenty-two legs received BMSC, while the other 22 legs received peripheral blood mononuclear cells (PBMC) as a control. Legs injected with BMSCs showed improvements in resting ankle-brachial pressure index (ABI), transcutaneous oxygen pressure (TcO_2), and rest pain, which established safety and efficacy of the protocol. Ischemic ulcers and gangrene improved in nearly half of all limbs. There was minimal improvement in ischemic limbs injected with PBMCs. Tateishi-Yuyama and colleagues concluded that the successful BMSC implantations were the direct result of increased EPCs, part of the $CD34^+$ fraction, and angiogenic factors released from the $CD34^-$ fraction. In a small 7-patient study in 2004, using the methods of Tatieshi-Yuyama [97], Higashi et al. [98] tested the premise that BMSC implantation would improve endothelium-dependent vasodilation in patients with limb ischemia [98]. They assessed the primary outcome by vasodilation response to Ach and SNP before and after implantation. Their results indicated that BMSC implantation increased the ankle-brachial pressure index, transcutaneous oxygen pressure, and basal leg blood flow. The BM-MNC implantation was composed of the $CD34^+$ fraction, which included EPCs and angiogenic growth factors such as VEGF. Thus EPCs augment new vascularization of ischemic tissue and repair fully differentiated endothelial cells that release nitric oxide, with increased endogenous angiogenesis. The authors propose that BM-MNC implantation could prevent the development of atherosclerosis via increasing endothelial function. Hernandez and colleagues [99] also examined BMSC implantation in severe unilateral lower limb ischemia patients. The authors recruited 12 patients to evaluate the efficacy and safety of autologous BMSC purified either by an automated method or by a manual procedure. Bone marrow was aspirated from the iliac crest and the BMSCs were

isolated by either a Fresenius AS 240 blood cell separator (Fresenius AG, Schweinfurt, Germany) or by Ficoll density gradient. The patients were monitored with ABI and arterial oxygen saturation (SaO_2), pain-free walking time, and resting pain scale evaluation. The two methods used for mononuclear cell separation gave results that were not significantly different ($P > 0.05$), though both groups showed significant improvements in resting pain as well as pain-free walking. The ABI and TaO_2 increased gradually following treatment. Nonetheless, limb salvage was achieved in 5 patients who had been advised to amputate. In 2008, Duong Van Huyen et al. reported a pathology study performed on four limbs amputated from patients transplanted with BMSCs to treat critical leg ischemia (CLI) [100]. The study reported for the first time active angiogenesis in BMSC-treated patients as compared to untreated patients. Angiogenesis was primarily located in the walls of distal arteries and veins. No angiogenesis was observed in tissues taken from the transplantation site. The authors note that since the proliferation lasted more than two months after the BMSC injection, that the stem cells could trigger a host self-sustained angiogenic response.

Intra-arterial injection

In 2011, Walter and colleagues reported a phase II, double-blind, randomized trial to treat 40 patients with BMCs or placebo delivered by intra-arterial injection [101]. At three months post transplant, there was no significant increase in the ankle-brachial index, thus the trial missed its primary endpoint. The authors reported; however, that there was significant improvement in ulcer healing and resting pain compared to the placebo group. There was no difference between limb salvage and amputation-free survival rates between the two groups. Interestingly, repeated BMC administration and higher BMC numbers and functionality were the only independent predictors of improved ulcer healing. The authors found that intraarterial injection of BMCs to be safe and feasible, and that for patients without extensive gangrene and impending amputation, the procedure accelerated wound healing. Concordant with those findings, Lawall et al. assessed the use of BMSC for the treatment of PAD and found autologous stem cell therapy to be promising for treating ischemic peripheral disease [102]. The authors reviewed clinical trials for outcome, and found that despite the small numbers of study subjects, different methods of cell isolation, variable dosages, and differences in degree of ischemia, that the results were markedly positive. Additionally, the treatments were well tolerated with few side effects. They cite a 2010 meta-analysis by Fadini et al., which found 108 PAD cell therapy trials, 37 of which were suitable for analysis [103]. The overall meta-analysis found BMSC therapy significantly improved ABI, TcO_2, rest pain, pain-free walking distance, ulcer healing, and limb salvage. Nonetheless, the authors argued that large randomized, placebo-controlled, double-blind studies are necessary and currently ongoing to provide stronger safety and efficacy data on cell-based therapy for cardiovascular disease.

Conclusion

Until recently, the differentiation capacity of MSCs was generally believed to be restricted to the three classical mesodermal lineages. However, recent data indicated that MSCs, when provided with the proper environment, can differentiate into additional cell types such as endothelial, pericytes, and smooth muscle cells, collectively contributing to vasculogenesis. Several per-clinical animal model studies demonstrated considerable therapeutic efficacy of infused MSCs in animals with various cardiovascular diseases. While the exact mechanism by which MSCs exert their function in tissue remodeling is not fully understood, several mechanisms have been implicated in this process including i) direct differentiation into endothelial cells, pericytes, and smooth muscles; ii) secretion of pro-angiogenic factors; iii) suppression of the inflammatory response and; iv) the expansion of endogenous CSCs. Inline of those pre-clinical studies, several clinical trials are currently underway to evaluate the therapeutic potential of human MSCs (hMSCs) in patients with various cardiovascular diseases. Published data from these clinical trials are very encouraging (Table 1). However, some of the remaining challenges in the field are how to isolate and expand hMSCs *in vitro* to obtain sufficient therapeutic number of cells, while maintaining their multipotency. Hence, more work is warranted in the area of characterizing novel surface markers for hMSCs, which might facilitate better expansion of those cells *in vitro* and lead to better therapeutic efficacy. Nonetheless, since there is increasing interest in the utilization of MSCs for regenerative medicine applications, the safety of administering MSCs to humans remains to be carefully evaluated, given the potential role of MSCs in driving tumorigenicity.

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