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Physiological conditions influencing regenerative potential of stem cells

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TABLE OF CONTENTS

1. Abstract
2. Introduction
2.1. Stem cells used for cardiac regeneration
3. Physiological conditions influencing stem cell regenerative potential of stem cell
3.1. Aging
3.2. Metabolic syndrome
4. Strategies to enhance stem cell regenerative potential
4.1. Pretreating stem cells before infusion
4.2. Treatment of the target tissue: how to rejuvenate cardiac tissue
5. Alternatives to autologous cell therapy
6. Conclusions and future perspectives
7. Acknowledgements
8. References

1. ABSTRACT

Stem cells are being used in the treatment of cardivovascular diseases. Here, we review the physiologic and pathologic conditions that impact the regenerative potential of stem cells in the treatment of cardiovascular diseases which include the influence of donor age and the presence of metabolic syndromes. We will also discuss strategies such as pretreatment of the recipient tissue or autologous or allogeneic stem cells by growth factors or drugs and by providing a synthetic scaffold and genetic modifications that impact the regenerative potential of stem cells. Finally, we will evaluate the current state of treatment of acute or chronic cardiovascular diseases with allogeneic stem cells.

2. INTRODUCTION

Ischemic heart failure is the leading cause of hospitalization and death in Western countries (1, 2). So far, the employment of current clinical therapies has been useful to slow the progression of cardiovascular diseases, however the regeneration of hearth tissue after an ischemic insult is still to be solved. Stem cell therapy has proven to be a promising option for the treatment of cardiovascular diseases of ischemic origin, by promoting cardiac tissue regeneration (3). Cardiac function and cardiomyogenic and vascular cell lineage differentiation have been improved by both bone marrow (BM)-derived and tissue specific stem cells in experimental models. Additionally, the capacity of stem cells to secrete paracrine pro-angiogenic and cardioprotective molecules has also been demonstrated. Although BM-derived cells are the most used cell type in cell therapy-based applications, non-BM or tissue specific stem cell types including mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs) and cardiac tissue derived progenitors (CPCs) are also under clinical evaluation.

A major limitation in the field of cell therapy is determined by the fact that the functionality of stem cells largely depends on the pathophysiological state of the donors. Metabolism is known to markedly influence stem cell fate and function (4). Indeed, aging, metabolic syndrome and diabetes represent major changes in metabolism that are associated with cardiovascular diseases and affect the quantity and quality of stem cells, thus limiting their therapeutic potential. In light of this, several research groups are currently attempting to further investigate this major issue. Presently, three main strategies have been proposed: 1) development of pre-conditioning treatments to restore or improve the function of 'sick' cells before their reinfusion, 2) preparation of the recipient tissue by treating the patient in order to increase the engraftment of transplanted cells, 3) use of allogeneic rather than autologous cells.

2.1. Stem cells for cardiac regeneration

Stem cells hold the ability to self-renew and to differentiate into multiple lineages according to their degree of stemness. Over the years accumulating evidences have suggested that almost all tissues (heart, adipose, brain, spleen, liver, kidney, lung, muscle, thymus,

pancreas, umbilical cord, placenta, dental pulp, skin and a variety of fetal tissues) retain niches of "resident" stem cells similar to those observed in BM (4,5). Consequently, the discovery of a cardiac stem cell (CSC) niche residing in the adult heart has changed the dogma that the heart is a post-mitotic organ devoid of regeneration potential (5). CSCs have been proven to self-renew, to possess clonogenic and multipotent ability and to differentiate both *in vitro* and *in vivo* into cardiomyocytes, smooth muscle cells and endothelial cells (5-10). Indeed, since the first discovery of c-kit$^+$ CSCs, different types of CSCs and cardiac progenitor cell populations have been identified both in rodents and humans (5, 9, 11), including a mixed population named cardiospheres containing c-kit$^+$ cells and cells expressing the stromal cell marker CD105 (12). Human c-kit$^+$ CSCs are the first cardiac resident population which has been used in a clinical trials (SCIPIO and CADUCEUS). The SCIPIO trial is a randomized phase I trial testing the safety and feasibility of using autologous c-kit$^+$ CSCs to treat ischemic cardiomyopathy. Despite the small number of patients enrolled, the trial has demonstrated that the infusion of CSCs is safe and feasible, reduces infarct size, improves regional and global LV function and the viability of the myocardium for up to 1 year after injection (13, 14). A second phase I trial named CADUCEUS, based on the use of human cardiosphere derived cells (CDCs), which contain c-kit$^+$ CSCs, has been conducted to improve safety and to verify the preliminary efficacy of intracoronary administration of autologous CDCs. The results of the CADUCEUS trial has proven that the use of CDCs is safe resulting in decreased scar size, increased myocardium viability and improved regional function of the infarcted myocardium 1 year post-treatment (15).

Despite this, it is hard to undoubtedly assess the best stem cell source to repair a damaged heart. In fact, besides CDCs and CSCs, other non-cardiac stem cell populations have been undergoing clinical evaluation. However, BM-derived cells (including HSCs, MSCs and EPCs) and adipose tissue derived mesenchymal stem cells (A-MSCs) still represent the main cell types that have been studied to regenerate a damaged myocardium.

Human HSCs, identified by the expression of surface markers such as CD133, CD34, or CD117 (c-kit), have been demonstrated to exhibit *in vitro* trans-differentiation ability towards myocardial lineages and therefore are intensively studied for the treatment of cardiac failure (16). Clinical trials conducted so far, using BM-derived HSCs have proved the safety and feasibility of this cell population for cardiac applications (8). Similarly, EPCs (another BM-derived stem cell population) have been extensively investigated for cardiac regenerative purposes (17). EPCs are angiogenic cells capable of differentiating into mature endothelial cells and promote the repair of damaged endothelium (18, 19). Several clinical trials have tested the use of these cells in the treatment of cardiovascular diseases. TOPCARE-AMI is the first randomized clinical study examining the use of EPCs for the treatment of acute myocardial infarction. This study has demonstrated that EPCs are able to enhance left ventricular ejection fractions and to reduce infarct size in the absence of cardiac hypertrophy (20); in a similar setting, REPAIR-AMI has demonstrated that transplantation of EPC abrogates early LV remodeling after AMI. The MAGIC and BOOST trials have shown regeneration and angiogenesis after G-CSF and intra-coronary BM-mononuclear cell infusion in patients with myocardial infarction (21-23).

An increasing body of evidence suggests that non EPC-like cells such as MSCs represents a promising stem cell source, particularly suitable for regenerative medicine applications. MSCs have been identified as a multipotent population showing plastic-adherent properties, ability to generate single-cell colonies (24) and mesodermal differentiation potential (chondrogenic, adipogenic and osteogenic differentiation) (25-27). To date, MSC phenotype cannot be identified by a specific single marker. Accordingly, in 2005 the International Society for Cellular Therapy (ISCT) published a position paper, defining the minimum criteria to define the MSC population; positive for CD73, CD90 and CD105 and negative for hematopoietic markers, such as CD45, CD34 and CD19 (28). Intriguingly, MSCs have been demonstrated to differentiate towards the cardiac lineage both *in vitro* and *in vivo* (26, 29-39) and the interest for this cell type is increasing due to their immune-modulating properties. Thus, MSCs are under investigation to test both autologous and allogeneic use in different clinical trials. Currently, autologous and allogeneic MSCs are examined in the POSEIDON-DCM trial as treatment options for non-ischemic heart failure.

Among the non BM-derived stem cells, a growing interest is focused on adipose tissue-derived stem cells (A-MSCs) described for the first time by Zuk *et al*. in 2001 (28). A-MSCs have been located in the vessel walls and perivascular niches (40) present in fat obtained during liposuction (a minimally invasive and low risk procedure). Interestingly, A-MSCs share many features with BM-derived MSCs. Indeed, with the exception of the surface marker CD34 (41), A-MSCs possess all the minimal criteria defined by ISCT for identifying MSCs (27, 28). Several studies have reported the ability of rabbit, mice and human A-MSCs to differentiate *in vitro* into cardiomyocytes under specific culture conditions (42-47). Moreover, in some cases, A-MSCs have also been shown to acquire pacemaker activity, although at low percentages (48). Similarly to BM-derived MSCs, A-MSCs improve cardiac function in rodent and pig models of MI, although it is unclear whether their beneficial activity is mainly the result of direct trans-differentiation into new cardiomyocytes, endothelial and smooth muscle cells or the indirect consequence of their paracrine action in

combination with their ability to exert a feeder-like support within the cardiac tissue (26, 49-54). Several phase I/II studies are currently ongoing to test the safety, feasibility, and efficacy of A-MSCs in cardiovascular diseases. APOLLO and PRECISE trials using freshly isolated A-MSCs have reported a trend for increased cardiac function in terms of perfusion, reduction of scar (APOLLO trial) and increased wall motion score index evaluated by magnetic resonance (PRECISE trial) (55, 56), whereas MyStromalCell Trial has tested the intramyocardial (IM) injection of culture-expanded A-MSCs stimulated with VEGF-A_{165} (57). The ADVANCE study was the first trial to evaluate the efficacy of A-MSC-based cell therapy in patients with AMI. Patients were treated or not with A-MSCs and the amount of infarct size reduction was assessed at 6 months (58). Unfortunately, the trial was terminated due to difficulties in recruiting patients and reaching the target enrollment of 216 patients which stood at 23 patients when the trial was terminated.

3. PHYSIOLOGICAL CONDITIONS INFLUENCING STEM CELL REGENERATIVE POTENTIAL OF STEM CELLS

There is compelling evidence that aging, metabolic and cardiovascular diseases are critically interconnected. Indeed, patients experiencing metabolic and cardiovascular diseases age faster. Furthermore, a clear association between cardiovascular risk and increased age has been largely reported (59). The average lifespan of humans is increasing, and the percentage of older people will continue to grow in the next 20 years. Therefore it is easily estimated that cardiovascular diseases will still represent the leading cause of death in people over 65 years of age (60). The scientific literature to date suggests that metabolism can profoundly influence the fate of stem cells (61-65). As a consequence, natural or pathological changes in metabolism associated with natural aging or with metabolic diseases of donors are predicted to negatively impact the quantity and quality of stem and progenitor cells, thus limiting their therapeutic potential (66-69).

3.1. Aging

Aging is a physiological process that affects all organs and tissues, leading to the deterioration of their normal function and to the inhibition or impairment of repair mechanisms. Indeed, the functionality of adult stem cells decreases with age which impact the normal tissue regeneration rate (68, 70,71-73). Myocardial aging has been proposed as an imbalance between the growth and death of myocytes, resulting in the accumulation of senescent myocytes with decreased contractile function (74). Cell senescence affects not only myocytes but also CPCs (74). In fact, cardiac aging correlates with a decreased number of active resident stem cells that display shorter telomeres, increased genomic instability, DNA damage, p53 activation and consequent cell cycle arrest (75, 76). Aged CPCs acquire the senescent phenotype and irreversible growth arrest demonstrated by the *in vivo* expression of the associated protein p16INK4a. Consequently, cardiac aging is associated with the formation of dysfunctional niches where an imbalance between dividing and senescent CPCs is observed, ultimately altering both tissue and organ homeostasis (77). Moreover, aging has been reported to influence tissue oxygenation by unbalancing the ratio between hypoxia (required for long-term preservation of undifferentiated CPCs) and normoxia (required for active cardiomyogenesis typical of young CPCs), thus exacerbating the accumulation of fibroblasts in hypoxic foci and increased plasma levels of the advanced glycated end product N'-carboxymethyl lysine (CML) (78).

Cardiac progenitor cells nested in this unbalanced and unfavorable microenvironment, are not able to re-enter the cell cycle (77). The decrease in tissue homeostasis results in the accumulation of senescent cardiomyocytes, thus compromising ventricular contractile function. In addition, aging negatively acts on CPC survival and migration, although the effect on growth is controversial (31, 35, 36). Confirmatory clinical studies have highlighted the poor efficiency in proliferation and differentiation of CPCs isolated from older donors compared to younger donors. Accordingly, the administration of old versus young cells in an animal model of MI, was associated with a reduced therapeutic potential of the treatment (33).

From a molecular standpoint, three main receptors appear to be involved in CPC senescence and the development of myocardial aging: IGF-1/IGF-1R, HGF-c-Met and the rennin angiotensin system (RAS). In the heart, activation of IGF-1/IGF-1R signaling is associated with CPC division, increased telomerase activity, prevention of replicative senescence and consequent better maintenance of the CPC pool (79-81). The expression of IGF-1R and IGF-1 synthesis are attenuated in the aged CPCs, negatively impacting telomere length and oxidative damage susceptibility (82). Indeed, IGF-1 is able to interfere with ROS generation and has been shown to decrease oxidative damage in the aging myocardium (77). ROS generation and accumulation of oxidative DNA damage in aged CPCs is associated with Ang II production (83) followed by the shortening and uncapping of telomeres (84). Aging also decreases Hepatocyte Growth Factor HGF production mainly affecting CPC migration ability, (in terms of the numbers and speed of CPC migration), by modulating their ability to move towards the damaged area and to promote heart regeneration (83, 85, 86). Despite this, BrdU incorporation experiments performed in the apex and atrium of senescent hearts revealed the presence of functionally competent CPCs which are responsive to growth factors and have preserved telomeres and are partially capable of reverting aging myopathy (83).

The influence of aging on stem cell function has also been reported in stem cell populations of non-cardiac-origin such as in A-MSCs. Aging affects the multilineage differentiation potential of A-MSCs as well as their proliferation capacity, cellular senescence and ability to produce paracrine factors. It has been shown that A-MSCs from aged patients have increased telomere shortening, decreased ability to stimulate the production of capillary-like tubes by endothelial cells and decreased secretion of pro-angiogenic factors such as VEGF, PGF, HGF, angiopoetin-1 and angiogenin. Furthermore, aging has been also associated with the abnormal expression of DNA damage repair genes and increased CHEK1 and p16INK4a which are involved in senescence (87, 88). Similarly, aging leads to a functional impairment of BM-MSCs, as shown by the limited ability to secrete angiogenic factors, reduced regenerative potential (88) and by the inability to induce neo-angiogenesis after MI (89). Moreover, cells from old patients exhibit reduced tolerance to ischemia, in terms of survival in ischemic muscle, compared to young patients (90).

Moreover, aging can impact the mobilization capacity of BM cells after acute myocardial infarction (AMI) in humans (91) resulting in a decreased number of $CD34^+$ cells in peripheral blood (92). Preclinical studies analyzing the angiogenic response of young cells in the aged heart, showed that the aged heart is rejuvenated when cardiac endogenous BM cells are replaced with young BM cells in a model of parabiosis (93). Telomere shortening and replicative exhaustion has also been observed in aged HSC populations (83, 94). HSCs display impaired migration and lineage commitment potential with decreased hematopoiesis and lymphopoiesis (83, 95-97). In particular, a similar impairment in CPC and HSC migration in response to specific stimuli, such as stromal derived factor (SDF-1) and VEGF (83, 97) has been observed. Aging has been associated with a reduction in the number and function of circulating EPCs (98). Furthermore, only EPCs from young patients are incorporated into the neovascolature and are able to restore angiogenic functions in the heart (99).

In conclusion, these observations clearly demonstrate that aging represents a critical factor influencing the regenerative potential of adult stem cells and therefore their possible use in cardiac regenerative medicine.

3.2. Metabolic syndrome

Metabolic syndrome includes several diseases such as insulin resistance, impaired glucose tolerance, type 2 diabetes, dyslipidemia, obesity, high blood pressure dyslipidemia, microalbuminuria, which are all associated with an increased risk of coronary artery disease (CAD) linked to aging (7, 100). Patients with metabolic syndrome are 3-4 times more likely to develop type II diabetes and the risk of developing cardiovascular disease is almost tripled (101). Mansilla *et al*., firstly proposed metabolic syndrome as an exhaustive syndrome of the stem cell pool, where defects in specific biological mechanisms of stem cells are strictly linked to the progression of the syndrome itself (102). In fact, the persistent inflammatory state (increased levels of IFN-α, IL-6) developed in metabolic syndrome leads to the continuous recruitment of the stem cell pool from their tissue of origin, thus causing pool exhaustion (102). Specifically, several evidences have highlighted a correlation between increased oxidative stress, telomere shortening and reduced function of circulating EPCs (103). Accordingly, a low percentage of circulating EPCs (40% less than controls without metabolic syndrome) was found in healthy men with abdominal obesity and metabolic syndrome (101). The decrease in the endogenous EPC pool observed in metabolic syndrome seems to accelerate the development of cardiovascular disease (102).

Metabolic syndrome also negatively impacts CPC activity and regenerative ability (104-106). One possible mechanism is related to the deregulation of intracellular Ca^{2+} levels due to the downregulation of Inositol 1,4,5-trisphosphate receptors (IP3Rs) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) which in turn influences both CPC proliferation and the overall regenerative capacity of the myocardium (107).

Among all conditions that contribute to the development of metabolic syndrome, type 2 diabetes is the most frequent. Interestingly, diabetic cardiomyopathy has been proposed as a CPC disease whose abnormalities lead to an imbalance between cell death and tissue regeneration (104). Noteworthy, cell death of mature cardiomyocytes, SMCs and ECs represent only a secondary phenomenon since CPC exhaustion precedes the progression of diabetic myopathy (104). Indeed, the metabolic pathways supporting CPC function has been reported only very recently. Salabei *et al*. have shown that murine CPCs expressing high levels of the glucose transporter Glut-1 largely depend on the extracellular glucose concentration for their glycolytic rate, thus suggesting that CPCs could be prone to hyperglycemic injury (40). Another protein playing an important role in diabetic myopathy is the p66shc protein, lack-of-which has beneficial effects on both viability and function of CPCs, and enhances cardiomyocyte differentiation (104). Hyperglycemia is also linked to O-glycosylation of proteins, including the activation of p53 which is able to up-regulate the renin-angiotensin system, conducive to the synthesis of angiotensin II (Ang II), resulting in increased cytosolic calcium-induced generation of reactive oxygen species (ROS) and apoptosis (108). ROS formation initiates DNA damage, telomere shortening, irreversible growth arrest and cell senescence (84). The impairment of the CPC pool in the heart due to hyperglycemia ultimately leads

to the accumulation of old and dying cardiomyocytes with consequent clinically relevant defects in contraction. Moreover decreased muscle mass and dilation of the heart chambers contributes to ventricular function deficits (104).

The relation between glucose and cardiac metabolism is extremely relevant. In the cardiac tissue, glucose metabolism plays a key role both in physiological and pathological conditions (109). For instance, the pentose phosphate pathway, is important for maintaining contractility and the redox state of cardiomyocytes, generating NAPDH which in turn acts as an alternative substrate to anabolic glycolysis. Importantly, CPCs isolated from hearts of diabetic mice followed by culture in high glucose media, display reduced activity in key enzymes of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase (G6PD) and trans-ketolase) resulting in the accumulation of glucose-induced Advanced Glycation End-products (AGEs), inhibition of pro-survival pathway Akt/Pim-1/Bcl-2 and activation of apoptosis (110). Similar phenomena also occur in CPCs isolated from diabetic hearts reconditioned with physiologically normal glucose levels, confirming the concept of the existence of a metabolic memory as a consequence of previous exposures to high levels of glucose (110). In human diabetic hearts, the number of CPCs is reduced also showing less proliferative and regenerative capacity compared to non-diabetic patients (110, 111). Cells derived from diabetic patients and cells preconditioned with high glucose exhibit reduced pro-angiogenic capacity that is restored following treatment with glyoxalase-1, suggesting that the defect can be linked to the accumulation of reactive dicarbonyls (111). Comparative studies have also shown that CSCs cultured in high glucose concentrations are still able to improve heart function in terms of LVEF but to a lesser degree than CSCs grown in normoglycemic medium (111).

Similar observations have also been reported in cardiac derived MSCs (C-MSCs). In a rat model of chronic myocardial infarction, C-MSCs have been shown to contribute to cardiac repair and display better persistence, migration and differentiation properties as compared to BM cells isolated from the same patients (112). Interestingly, C-MSCs derived from patients with diabetes Mellitus (DM C-MSCs) have shown a reduced rate of proliferation, premature senescence (identified by the positive staining for beta-galactosidase and increased p21wa61 protein expression, respectively). Furthermore, diabetic C-MSCs display a decreased ability to differentiate along adipogenic, endothelial and cardiac lineages when compared to C-MSCs isolated from normoglycemic patients (113).

Long-term defects in type 2 diabetes have also been associated with damage in A-MSC function. Cramer *et al*. have shown that high levels of glucose reduce the proliferative capacity of A-MSCs isolated both from diabetic (dA-MSCs) and non-diabetic (nA-MSCs) donors, however the influence was more significant in dA-MSCs. This phenomenon seems to be reversible only in nA-MSCs, where there is an increase in proliferative capacity after insulin treatment, unlike the dA-MSCs that instead showed increased senescence and apoptosis. Furthermore, elevated glucose levels influence A-MSC differentiation, reducing the osteogenic and chondrogenic potential and increasing the adipogenic commitment (114).

A similar scenario has been demonstrated in other stem cell types. For instance, both type 1 and type 2 diabetes are associated with reduced levels (mean reduction of 40%) and poor functioning circulating EPCs (115-117). The number of EPCs inversely correlates with glycated hemoglobin (HbA1c) levels (118-120) and with fasting glucose levels. In particular, a decreased number of $CD34^+$/KDR EPCs have been reported to inversely correlate with the severity of diabetic vasculopathy (121). EPCs from patients with type 2 diabetes mellitus (DM), have shown impaired proliferation (important for amplifying the pool of endothelial cells), tubulization (necessary to create new vascular structures), adhesion to activated endothelium (critical for the recruitment of circulating EPCs to the site of interest) and migration toward cytokine gradients (such as G-CSF). Interestingly, these defects are not reverted by normoglycemic cell culture conditions (122, 123). However DM does not impact different properties of EPCs such as adherence to fibronectin, collagen and quiescent endothelial cells (67, 116). More importantly, the hyperglycemic-induced alterations in EPC function are mediated by pathophysiological mechanisms such as inflammation, oxidative stress, altered Akt pathway signaling and nitric oxide (NO) have been reported on multiple levels: mobilization from BM, trafficking and survival in the circulation, homing and neovascularization (67). From a molecular standpoint, a possible explanation of EPC defects could be the activation of MAP kinases, such as p38 that decrease the proliferation and differentiation capacity of EPCs in culture (124), increasing EPC senescence and apoptosis. In DM, increased levels of SDF-1 in the BM (due to increased CD26/DPP-4) and decreased SDF-1 production in the ischemic tissue is conducive to a decrease in the SDF-1 gradient which is normally responsible of mobilizing EPCs from the BM into the circulation (125-127). It has been shown that the long-term *in vitro* exposure to high glucose concentrations, inhibits the proliferative capacity, EPC migration and the capability of colony formation (128, 129). In these conditions a decrease in NO and vascular endothelial growth factor (VEGF) secretion in combination with a reduction in the activity of superoxide dismutase (SOD) have been found. Therefore, it is clear that the

defects observed in diabetic EPCs are certainly due to the toxic effects of high glucose. However, strict glucose control in DM patients had no significant effect on the rates of major cardiovascular events, death, or microvascular complications (130-132). When used in animal models of ischemia, diabetic-EPCs were less effective in restoring blood flow compared to cells derived from healthy controls (124). In addition, in diabetic mice, injection of BM-MNCs from healthy controls was more effective than BM-MNCs from diabetic patients. As observed in EPCs, BM-MNC dysfunction has been associated with an *in vitro* impairment of differentiation capacity and *in vivo* defects in the formation of vascular-like structures in Matrigel® plug experiments (133). Moreover diabetic BM-MNCs are not able to improve cardiac function following injection into a mouse model of MI unlike control BM-MNCs (134).

4. STRATEGIES TO ENHANCE STEM CELL REGENERATIVE POTENTIAL

The literature reviewed in the previous sections clearly indicate that factors such as age, metabolic syndrome and diabetes negatively influence the regenerative capacity of stem and progenitor cells used for cell therapy. The development of innovative approaches to improve the engraftment and regenerative potential of stem cells is a critical challenge for the successful employment of cell therapy in the cardiovascular context. To remedy this, three main strategies have been proposed: 1) the development of treatments to restore or to improve the function of 'sick' cells before their reinfusion in the damaged heart, 2) the preparation of the recipient tissue by treating the patient with drugs able to increase the engraftment of transplanted cells, 3) the infusion of allogeneic rather than autologous cells.

4.1. Pretreating stem cells before their reinfusion

The rationale of this approach consists the activation or enhancement of specific molecular pathways able to improve the response of stem cell progenitors to stress or enhance their regenerative abilities following the *in vivo* injection in the damaged tissue. Stem cells are commonly treated after their isolation, during *in vitro* expansion and before their injection into the site of injury. In this section we will review some of the most representative examples of cell pretreatment-based protocols that are currently employed including: growth factors, drugs, viral transduction and tissue engineering based approaches.

Statins are drugs that inhibit the synthesis of endogenous cholesterol by acting on the hydroxylmethylglutaryl CoA-reductase. Several studies have suggested that statins benefit patients with ischemic and non-ischemic cardiomyopathies (135). Statins exert pleiotropic effects with different outcomes depending on the target cell, the type of statin, and the concentration and timing of treatment. Most of the studies regarding the effect of statins on stem cell biology have been performed on EPCs and MSCs. Systemic administration of statins has been shown to normalize EPC levels, restore their endothelial regenerative capacity and reduce the cardiovascular risk in subjects with metabolic syndrome (101). Statins increase the bioavailability of NO and reduce EPC apoptosis and oxidative stress (136). Moreover, statin treatment delays EPC aging by increasing the expression and activity of the telomere-capping protein TRF2 and cell cycle cyclins, and decreases the cell cycle inhibitor p27Kip1 (137, 138).

Another treatment able to improve EPC function is represented by the administration of growth factors. Insulin growth factor 1 (IGF-1) increases the number of circulating EPCs, enhancing their colony formation ability, migratory capacity and the incorporation into tubular-like structures (139). Notably, either alone or in combination with hepatocyte growth factor (HGF), the IGF-1 treatment has been also proven to induce a beneficial effect, by acting on CPC division and differentiation after injection in a rat model of MI (83). Similarly, when administrated systemically, stem cell factor (SCF) has been shown to partially activate CPCs *in situ* reversing myocardial aging in mice (77). Additionally the administration of Placenta Growth Factor (PlGF) has been shown to increase the *in vivo* mobilization and recruitment of BM derived stem cells in a diabetic murine model of hindlimb ischemia. PlGF also improves EPC differentiation both *in vitro* and *in vivo,* increasing neovascularization and blood flow recovery in treated *versus* untreated diabetic mice (133). The decrease of endothelial nitric oxide synthase (eNOS) observed in diabetic EPCs has been shown to be rescued by hyperoxia treatment, or by SDF-1 administration, which is able to increase the neovascularization and wound healing in animal models (70, 140). Insulin in diabetic patients can increase the number of circulating EPCs, to boost their *in vitro* growth and angiogenic potential, VEGF production and mobilization (125). This effect is enhanced in the presence of the SDF-1 3'-A/G allele, a polymorphism in SDF-1 gene associated with increased EPC mobilization (141).

A possible alternative to growth factors is represented by the use of drugs, reported as particularly beneficial to 'correct' the detrimental influence that metabolic syndrome and/or diabetes have on stem cell function. Benfotiamine (BFT), a synthetic S-acyl derivative of thiamine (vitamin B1), has been shown to reactivate the pentose phosphate pathway, which is reduced in the diabetic context, restoring the function of CPCs in the heart (110). Similarly, the histone acetylase (HAT) activator pentadecylidenemalonate 1b (SPV106), has been shown to help the function of diabetic C-MSC, improving their proliferation, differentiation capacity and histone modifications which are compromised in patients

with type 2 diabetes (113). Methylglyoxal (MG), that is a reduced derivative of pyruvic acid, accumulates in diabetes and impairs neovascularization. The overexpression of MG-metabolizing enzyme glyoxalase-1 (GLO1), is able to revert the detrimental effects of diabetes in BM-cells (142) and CPCs (111), restoring their proangiogenic capacity. Other drugs can act by amplifying the number and function of EPCs in type II diabetes; Angiotensin II type 1 Receptor Blockers ARBs increase the number of EPCs (143); agonists of PPAR-γ act through the PI3K/Akt pathway (144); the thiazolidinediones activate the Akt/eNOS pathway; ephrin-B2/Fc (145), and DDP-4 inhibition augments the *in vitro* number (146), migration capability and regenerative capacity of EPCs (147, 148); whereas G-CSF (149), erythropoietin (150), estrogen (151) and VEGF (152) act as mobilizing agents (153). Hydrogen sulfide, is able to enhance wound healing in type II diabetes patients by restoring EPC function and increasing the levels of angiopoietin-1 (154).

Different strategies based on genetic or molecular modifications of stem cells have also been proposed. For instance, the inhibition of p38-kinase by SB203580 (a pyridinyl imidazole inhibitor of p38-kinase) or by infection with a dominant negative p38-kinase adenovirus or adiponectin (155) is able to act on EPC proliferation and differentiation in diabetes. Indeed, inhibition of p38 promotes the differentiation of EPCs to the endothelial lineage rather than the monocyte-macrophage lineage and it increases VEGF levels which is largely demonstrated to play a key role in angiogenesis (70, 124). Alternatively, the molecular regulation of EPC function, which is physiologically compromised in diabetic patients, may reside in the regulation of miRNA 126 and 130a, normally altered in diabetes (156, 157) and recently proposed as a novel therapeutic target.

Interestingly, the strategy of pretreating stem cells with drugs to improve stem cell regenerative potential has also been successfully assessed in A-MSCs. Natural agents such as curcumin, extracted from the spice turmeric has been reported to exhibit antioxidant and anti-inflammatory properties (158, 159). In a rat model of myocardial ischemia-reperfusion injury (IRI), transplantation of curcumin pretreated A-MSCs not only improves heart function, higher cell retention, smaller infarct size, but also decreases myocardial apoptosis and promotes neovascularization and increased VEGF levels in the peri-infarcted area (160).

Finally, a triple approach based on the combination of tissue engineering, stem cells and drugs is now under clinical evaluation, such as the use of scaffolds to enhance stem cell engraftment. A-MSCs embedded in a scaffold of platelet-rich fibrin (PRF) have been shown to significantly promote angiogenesis, to preserve wall thickness and heart function, as well as to reduce infarct size, LV chamber size, and LV remodeling (161). Similarly, the use of alginate hydrogels has been demonstrated to augment the retention of A-MSCs after their injection in patients with ischemic heart disease without affecting their paracrine potential (162). The use of A-MSCs conjugated with VEGF-releasing poly lactic-co-glycolic acid (PLGA) and pharmacologically active microcarriers (PAMs) has resulted in their increased proliferation, angiogenic differentiation and VEGF production, as well as in decreased cell death compared to unconjugated A-MSCs. Similarly, A-MSCs conjugated with VEGF-releasing PAMs could have therapeutic applications in treating patients with vascular disease, including those with a previous AMI (163). In fact, in a murine model of AMI, the transplantation of A-MSCs conjugated with VEGF-releasing PAMs into the ischemic region has already been shown to improve post-AMI cardiac function and to increase arteriogenesis. Lastly, a significant improvement of the efficacy of stem cell regenerative activity has also been demonstrated using hydrogels suitably modified, in order to allow the controlled release of NO by MSCs, thus enhancing their therapeutic efficacy in the treatment of MI (164).

4.2. Treatment of target tissue: how to rejuvenate cardiac tissue

As already discussed, aging and metabolic dysfunctions have a detrimental effect not only on stem cells but also on tissue function, highlighting the critical role of the cardiac microenvironment in the recipient. The biological and molecular alterations within a hostile microenvironment such as that produced after a cardiac insult, may certainly influence the proliferative capacity of stem cells once injected (70, 162, 163). Therefore, a key question currently under clinical evaluation is the possibility to rejuvenate the target tissue (68). Accordingly, changing the microenvironment of the injection site may be useful to increase the engraftment of transplanted cells, but also their regenerative capacity has been targeted. It has been demonstrated in a model of hetero-chronic parabiosis, that circulating factors can “rejuvenate” hMSCs in the old parabiont (71). Specifically, the factors that seem to hold rejuvenating properties on aged hMSCs are linked to the circulating growth differentiation factors (71), including members of the transforming growth factor beta superfamily (VEGF and bFGF; 72, 73). These effects have been demonstrated both *in vitro* and *in vivo* after MI. To date a variety of factors delivered to the site of cell therapy injection locations have been exploited. Local injection of HMGB-1 increases the regeneration of infarcted myocardium by activating endogenous CSCs (165, 166). Transplantation of syngeneic cardiac fibroblasts stably overexpressing SDF-1, increases the homing of c-kit$^+$ cells into the myocardium, which has also been shown to generate a stabilized SDF-1 mutant (167, 168). The overexpression of IGF-1 restricted to myocytes retards both aging of CPCs and myocytes, and the onset of

ventricular dysfunction (80). It has been demonstrated that age-dependent impairment of EPCs is corrected by growth-hormone-mediated IGF-1 increase, both in a preclinical animal model and in humans (139). Moreover, the delivery of IGF-1 injected locally into the myocardium through biotinylated peptide nanofibers, improves neonatal rat cardiomyocyte cell therapy after MI (81). The administration of PDGF, which is downregulated during aging, is able to reverse the senescent predisposition to increased cardiac injury (169). Other biological candidates have been recently discovered. For instance, the circulating extracellular domain of Klotho, a transmembrane protein that controls the sensitivity of cells to insulin which plays a main role in aging, has been postulated to act as an anti-aging hormone (170, 171). The overexpression of Klotho in mice extends lifespan (170) not by simply acting on insulin/IGF-1 signaling, but also by increasing oxidative stress resistance *via* the the upregulation of manganese superoxide dismutase and also acting as an essential cofactor of fibroblast growth factor signaling (172). Additionally, Klotho can influence the regenerative response of tissue-resident stem cells in multiple organs involving a more complex scenario. Finally, a novel rejuvenating approach consists low-energy shock wave-induced tissue activation which stimulated the expression of SDF-1 and VEGF in the target tissue and promoted homing of intravenously infused EPCs in uninjured and chronically ischemic rats (173).

5. ALTERNATIVES TO AUTOLOGOUS CELL THERAPY

So far, cell therapy (not only in the cardiovascular field), has been mainly based on the employment of autologous stem cells, treated to enhance their potency or commitment in order to replace or regenerate the damaged tissue. Given the role of aging in recipients (69), the host environment plays an even more relevant role in the clinical outcome of the cell regenerative response to the ischemic injury.

Over the years autologous cell therapies have shown several limitations. The proliferative and regenerative capacity of progenitor cells decreases with age (174, 175), with a consistent reduced efficacy of progenitor cells from old patients. Another practical limitation consists in the collection of BM samples or biopsies from other sources that often require invasive procedures. As the low percentage of stem and progenitor cells in the starting material might occur, cells need to be expanded *ex vivo* before autologous applications. Such procedures may require days (176) or weeks (177), depending on the cell number needed and the age and comorbidities of the donor, as progenitor cells from old patients have reduced proliferation potential (90, 174, 175). To date, stem cell *ex vivo* expansion requires Good Manufacturing Practice (GMP) compliant facilities. Although safety and reproducibility is assured within cell factories, however, patient-specific tissue harvesting and cell preconditioning may result in a delayed start of the therapy, with a risk of the introduction of possible variations in cell potency related to patient age and disease (68, 178, 179).

One possibility to improve the efficacy of stem cell therapy is represented by the use of allogeneic cells. Allogeneic cell therapy from young donors have several advantages over autologous, including a higher regenerative potential (180) and availability for clinical use at any time. Moreover, cells from a single donor can be used for many patients, thus tremendously reducing the manufacturing costs of cell products. In this scenario, we could hypothesize the employment of young cells in aged patients or healthy cells in patients with metabolic diseases (such as diabetes or metabolic syndrome) to overcome the decreased activity of the respective autologous cells.

Allogeneic CDC transplantation without concomitant immunosuppression has been demonstrated to be safe (181, 182) and promotes cardiac regeneration, improving heart function and endogenous repair mechanisms in a rat MI model (178). CDCs have a low immunomodulatory profile and their allogeneic transplantation only induces a transient mild local immune reaction in a rat MI model. The biological and functional benefits of such a strategy, almost indistinguishable from syngeneic transplantation, could persist 6 months post-MI (178).

In addition, MSCs have been shown to inhibit T-cell responses through the modulation of B cells and antigen-presenting cells (183-185), to induce a local immunosuppressive environment through the production of prostaglandins and anti-inflammatory cytokines (186, 187), and to inhibit lymphocyte proliferation, dendritic cell maturation and alloimmune rejection (186, 188-193). Due to their immunomodulatory properties, MSCs have been tested in initial clinical studies of graft versus-host diseases (194), osteogenesis imperfecta (195), glycogen storage diseases (196), Crohn's disease (197) and organ transplantation (26, 27, 198). In fact, adult human MSCs express intermediate levels of MHC-I but do not express human leukocyte antigen (HLA) class II (16) or co-stimulatory molecules on the cell surface under normal *in vitro* conditions. However, HLA-II has been detected in lysates of unstimulated MSCs, presumably reflecting intracellular deposits of the antigen. Treatment with interferon-γ has been found to induce the expression of HLA class II on the cell surface (189). Another study has shown that MSCs express MHC-II as antigen presenting cells in presence of low levels of interferon-γ, whereas high levels of interferon-γ impair its expression (199).

Despite this, the potential immunogenicity of allogeneic MSCs remains an unresolved issue that may affect their clinical efficacy. In fact, it has been shown that although MSCs actively inhibit rejection (200), they might be immune-rejected upon cardiac differentiation in the injured heart (201). Again, both allogeneic MSCs and autologous cells have been shown to improve cardiac function 1 month after MI in a rat MI model (201). However, 6 months after infarction, the allogeneic MSCs were rejected, resulting in decreased heart function. If MSCs were *in vivo* pretreated with prostaglandin E2, allogeneic injection of MSCs still prevents rejection and ventricular function could be restored, thus highlighting the importance of the immune/inflammatory system in balancing cardiac repair (202).

The first Phase I, randomized, double-blinded, placebo-controlled, dose escalation clinical study (181), has demonstrated that delivery of MSCs is safe and elicits no allogeneic T-cell response. This trial has evaluated the effect of cell dosage, also showing that higher doses of stem cells may not always provide greater benefit (203 205). Authors have postulated that the biological effect of stem cell injection likely reaches a plateau, as lower cell doses have been found to display greater improvements in heart function, whereas higher cell concentrations could result in improved inflammatory responses, thus affecting cell performance (203). The POSEIDON trial has randomized 30 patients with heart failure to receive either allogeneic or autologous mesenchymal stem cells (MSCs) by direct endomyocardial injection. Several studies have shown delayed host responses to allogeneic MSCs *in vivo* around 2 weeks after transplantation, suggesting that tolerance of MSCs across the allogeneic barrier may not be absolute (201, 206-208). Moreover, the trans-differentiation of MSCs into cardiac lineage cells in response to 5-azacytidine or cytokine treatment increased the expression of immunostimulatory MHC-Ia and MHC-II molecules and decreases the immunomodulatory MHC-IIb, resulting in enhanced cytotoxicity of MSCs in co-culture with allogeneic leukocytes (201). These observations suggest that MSCs can switch their immune states from immunomodulatory to immunostimulatory depending on the biological context (201).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

It is hard to predict whether or not stem cell-based therapies will represent a clinically reliable therapeutic strategy to fully restore cardiac function., The impact of diseases such as metabolic syndrome, diabetes and aging on endogenous stem/progenitor cells and on cardiac microenvironment may limit the benefits of cell therapy.

So far, cell-based therapy has been extensively studied in animal models, using the abovementioned cell populations. Most of the clinical trials have only tested the safety and efficacy of autologous adult stem cells. A progressive research approach in preclinical and clinical studies is needed to ensure the realization of cardiac regeneration by allogeneic cell therapy. The allogeneic scenario may offer the advantage of collecting young and healthy “universal donor cells” (209), and to expand the cells in advance in dedicated and controlled GMP-facilities. This would allow the potential to obtain an unlimited number of doses for clinical administration and even to treat acute diseases such as MI. Additionally, the employment of a specific stem cell population showing low immunogenic properties would avoid the use of immunosuppressive treatment. To date, several issues remain unresolved. Experimental evidence suggest that even *in vivo* allogeneic transplanted MSCs may switch to different immune states, inducing alloimmune rejection (201). Therefore, minimizing donor-recipient HLA-II mismatch might represent a good strategy in order to attenuate alloimmune rejection of transplanted MSCs. In line with this, very promising studies are focusing on the establishment of a GMP HLA homozygous haplobanks which should overcome the issue of HLA mismatch in the context of cell-based transplants (210).

In addition, the low retention and engraftment of stem cells within damaged cardiac tissue after clinical administration requires a better definition of the biological mechanisms underlying such processes. Nevertheless, given the key role of stem cell paracrine action as a major mechanism involved in immune rejection after *in vivo* injection, a long-term survival of transplanted stem cells may not be necessary to achieve long-lasting clinical effects (211). In the future the “off the shelf” cell therapy product could become a reality, by developing human pluripotent stem cell bio-banks with low immunogenic properties and high regenerative capacity (212).

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