Mesenchymal stem cell-derived exosomes: A novel potential therapeutic avenue for cardiac regeneration

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Abstract: Coronary artery diseases (CADs) represent a significant cause of death worldwide. During recent decades the rate of cardiovascular mortality has been declined as a result of modern medicine and surgery. However, despite the fact that cardiac cells, including cardiomyocytes (CMCs), vascular smooth muscle cells (VSMC) and vascular endothelial cells (VEC), can be regenerated by cardiac adult stem cell, the regenerative capacity of these cells is limited and inadequate to functionally regenerate heart damaged tissue. Thus, growth reserve of the heart fails to restore the structural integrity of the myocardium after infarction and healing is associated with scar formation. An explanation for this is that cardiac reside stem cells are present throughout the infarction site but die rapidly by apoptosis. Furthermore, microenvironment surrounding the damage site is not promising for the cells survival and renewal. Hence, recent advances in the stem cell therapy have emerged as an attractive approach to replace the lost cells. In this context, mesenchymal stem cells (MSCs) has considered as one of the most promising candidates for regeneration of cardiac cells, lost upon injury. The regenerative capacity of MSCs has primarily been centered on the hypothesis that these cells would engraft, differentiate and replace damaged cardiac cells. However, experimental and clinical observations so far have failed to establish if this differentiated is considerably relevant to MSCs cardiac regenerative properties. Recent reports have suggested that these therapeutic properties, at least in part, are mediated by paracrine factors released from MSCs. This review provides a concise summary of current evidences supporting the paracrine hypothesis of MSCs. In particular, the scope of this review focuses on the role of MSC-derived exosome (MSC-EXs) as a therapeutic modality for the treatment of CADs, particularly ischemic myocardial dysfunctions.

Key words: Coronary artery diseases, cardiac regeneration, mesenchymal stem cells, exosome.

Introduction

Coronary artery diseases (CADs), also known as ischemic cardiac disease (ICD) account for >17 million deaths globally each year (30% of all deaths) (1). It is clinically manifest as acute myocardial infarction (AMI), stable angina, unstable angina and sudden cardiac death (SCD). The underlying pathology of CADs is formation of plaque within coronary artery - a condition called atherosclerosis - that leads to blockages of blood flow. Regional myocardial ischemia, subsequent to a significant reduction or cessation of coronary arterial blood flow, induces CMCs loss in the segment supplied by the coronary artery.

Unfortunately, myocardial infarction (MI) endogenous cardiac repair via differentiation of residing cardiac progenitor cells (CPCs) or renewal of pre-existing adult CMCs appears to be limited by insufficient number of these cells. Moreover, although many life-saving cardiovascular therapies such as pharmacotherapy and surgical treatments are widely available, they all fail to restore or regenerate damaged cardiac tissue. Thus, the fact remains that after a loss of myocardial cells, the resulting necrotic and scar tissue cannot be restored properly. Therefore, the ideal therapeutic approach would aim to replace the lost cells and restore the cardiac normal function.

Stem cell-based regenerative medicine represents a new paradigm approach in treatment of cardiac injuries. Currently, several types of stem cells, including cardioid-derived stem cells (CSCs), bone marrow-derived stem cells, MSCs, skeletal myoblasts (SMs), and hematopoietic stem cells (HSCs) have been applied in clinical researches (2-4).

MSCs have been shown to be a stem cell population with great promise for cardiac regenerative applications (5). Several preclinical and clinical studies have supported the hypothesis that cardiac transfer of MSCs can have a favorable impact on cardiac function after injury (6, 7). The initial focus of therapeutic effects of MSC was based on their ability to differentiate into multiple cardiovascular cell lineages including VEC, VSMC and CMCs (8-10). Basically, the expectation was that upon implanting or injecting MSC, the cells would colonize at the lesion site and differentiate to appropriate cardiovascular cells. However, recently this mechanism has been challenged as MSCs do not persist well inside ischemic microenvironment of injured tissues and if there is low or no incorporation into the host tissue, most of the cells are lost within a month (11). Furthermore, no direct in
vivo evidence was obtained in support of differentiation of MSC to cardiovascular cells at the site of injury (12). In conclusion, MSCs differentiation into functional cardiovascular cells seems to be inefficient solely to exert therapeutic effects on extensive cardiovascular injuries resulted from ischemia.

In recent years it has been suggested that MSC produce components with biological activities that appear to mediate their therapeutic effects. This so called “paracrine hypothesis”, has gained much attention and is greatly supported by experimental evidences (13-17). Even though, the initial attention on paracrine therapeutic potential of MSC had centered on factors such as chemokines, cytokines and growth factors, none could sufficiently explain the efficacy of MSCs against the complex pathological processes of cardiac injury. Thus, recent efforts to investigate the therapeutic properties of MSCs secretome have shifted from these molecules to more complex structures, known collectively as extracellular vesicles (EVs).

EVs consist of several classes of secreted vesicles such as exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles and apoptotic bodies. Among EVs, exosomes are, by far, the best characterized for their potential utility as cell-free therapeutic candidates that can mediate cardiac regeneration.

In the present review we will provide a brief discussion about MSCs cell-based therapy for cardiac regeneration followed by a deeper analysis of MSCs paracrine effects, with central focus on exosomes in this regard.

**Administration of mesenchymal stem cells in cardiac regeneration**

Despite recent studies, suggesting that the heart has intrinsic mechanisms of self-regeneration following loss of cardiovascular cells, unfortunately after a major tissue injury, it cannot regenerate itself to the optimal level. In clinical practice, the concept of regenerative medicine and tissue repair using stem cells has increased considerably in the last decade. In the context of cardiac regeneration, the ultimate goal is to restore perfusion, contractility, lusitropy and conduction by repopulation of injured tissue with normal and healthy cardiac cells. Across different types of stem cells, MSCs are indicated to be a promising stem cell candidate for repairing cardiac tissue damages (8, 18, 19). They can be easily isolated from bone marrow, adipose tissue, lung, liver, tendons, placenta, amniotic fluid, dental pulp, synovial membrane and heart (20, 21). The advantages of MSCs in clinical use are due to their easily isolation, multilineage differentiation potential, low immunogenicity, immunosuppression effects, low tumorigenicity and lack of ethical controversy.

Cardiac repair after MSC transplantation is well described in both preclinical models and clinical trials (22). Moreover, in a recent phase I/II randomized, double-blind, single-dose study, the intravenous administration of allogeneic MSCs in patients with MI was reported to be safe and well tolerated (6).

Available data suggest multiple beneficial effects of MSCs in cardiac regeneration, which together lead to the repair of scarred or dysfunctional cardiac tissue (23). Currently four mechanisms are suggested to be involved in MSCs cardiac repair properties. **Differentiation of MSCs to cardiovascular cells**

The ability of MSCs to differentiate into cardiovascular cells including CMCs, VSMC, and VEC (24, 25) is subjected to controversial results. In 2001, Toma et al (26) reported that only a limited number of human bone marrow derived MSCs (BM-MSCs), injected into the left ventricle of immunodeficient adult murine, were engrafted within the myocardium (0.44%) and differentiated into the CMCs. In other study, Quevedo et al. (27) reported that only a small number of allogeneic MSCs can engraft, survive and differentiate to into CMCs, VSMC, and VEC in a swine model of chronic ischemic cardiomyopathy. In contrast to available reports of rare engraftment and differentiation of MSCs, some other studies indicated no engraftment of the MSCs injected within the cardiac borderzone in animal model of cardiac injury (28). It is suggested that, due to ischemic microenvironment of injured tissues that poses a serious problem for MSCs survival, these cells do not persist for long inside the environment of damaged myocardium (11). In fact, the regenerative efficacy of MSC transplantation in repairing tissue damages has been increasingly observed to be dependent on other mechanisms rather than engraftment and differentiation.

**Cell-Cell interactions and CSCs stimulation**

The ability of MSCs to stimulate proliferation of endogenous CSCs and restore the functional CMCs has been indicated in both animal model of MI and coculture experiments with CSCs and MSCs (29). In this regard, cell-cell interactions are suggested to play an important role in CSCs stimulation by MSCs. However, other available studies have reported that stimulation of endogenous CSCs via MSCs is infrequent or absent in vivo (30), which is due to the fact that ischemic microenvironment is a major obstacle for MSCs physical interaction with cardiac resident cells (19, 30, 31).

**MSC and CMCs fusion**

Fusion of MSCs with adult cells, including CMCs, has been proposed as a regenerative approach. However, available studies report that this phenomenon is a rare and inefficient process to cope with the major loss of CMCs after MI or other ICD, ruling out any significant involvement in MSCs cardiac regeneration effects (19, 30).

**Paracrine signaling**

Following indication of transient engraftment, low levels of MSCs stimulation of CSCs and infrequent MSCs fusion with CMCs, other possible cardiac regeneration mechanism remains to be MSCs paracrine effects (13).

Currently paracrine effects are implicated as the most comprehensive and enduring mode of action for MSCs therapeutic modality in cardiovascular repair. MSCs are known to secrete a wide array of cytokines and growth factors, which can suppress the immune response, inhibit fibrosis and cell apoptosis, enhance angiogenesis, activate cell proliferation, and stimulate differentiation of tissue specific stem cells. This complex secretome of MSCs is released either directly or packaged in EVs.
Exosome formation is a fine-tuned process which includes four stages: initiation, endocytosis, multivesicular bodies (MVBs) formation, and exosome secretion (Figure 1). MVBs which appear along the endocytic pathway are formed by the inward budding of an endosomal membrane into the lumen of the compartment, thus, these vesicles are characterized by the presence of vesicles in their lumen. After vesicular accumulation, the MVBs are either sorted for cargo degradation in the lysosome or released into the extracellular space as exosomes by fusing with the plasma membrane. The mechanisms underlying cargo clustering into the intraluminal vesicles (ILVs) are not yet fully elucidated. Both endosomal sorting complex required for transport (ESCRT)-dependent and independent signals have been suggested to determine the sorting of Exosomes (38). Moreover, the syndecan heparan sulfate proteoglycans and their cytoplasmic adaptor syntenin have been indicated to control the formation of exosomes (38). The Rab guanosine triphosphatases (GTPases) have been found to critically regulate exosome secretion.

The function of the original cell from which an exosome originate can be deduced by the makeup of proteins, lipids, miRNAs, mRNA, non-coding RNAs and other molecules found in a particular exosome. Both ubiquitous and cell-specific membrane molecules (lipids and proteins) and loaded cargos (proteins, mRNAs, non-coding RNAs) are targeted selectively to exosomes. The most common proteins, mRNA, and miRNAs found in exosomes have been deposited in ExoCarta (www.exocarta.org). The current version of ExoCarta hosts 41,860 proteins, >7540 RNA and 1116 miRNAs found in exosomes have been deposited in ExoCarta (www.exocarta.org). The current version of ExoCarta hosts 41,860 proteins, >7540 RNA and 1116 miRNAs have been deposited in ExoCarta (www.exocarta.org). The current version of ExoCarta hosts 41,860 proteins, >7540 RNA and 1116 miRNAs have been deposited in ExoCarta (www.exocarta.org).

At present, the most commonly used methods for exosome isolation include ultracentrifugation, combined with sucrose gradient, and the immune-bead isolation (e.g., magnetic activated cell sorting; MACS). There are many commercial kits available for the extraction of exosomes. Transmission electron microscopy (TEM),
Western blot, and FACS are frequently used to characterize the isolated exosomes based on their biochemical properties (e.g., morphology, size, exosomal markers).

Identification of MSCs secretion of exosomes has led to a growing body of studies on the therapeutic applications of these particles in regenerative medicine. Currently, MSCs-EXs derived from different cell sources have been used for regeneration of acute kidney injuries (39), skin wounds (40), liver-injury (41), traumatic brain injury (42) and myocardial damages (43). Following the identification of the role of exosomes in cardiac repair, growing body of researches investigated the regenerative and protective mechanisms of these nanovesicles (Table 1 and Figure 2).

Administration of exosomes has different advantages over stem-cell transplantation. Occlusion in the distal microvasculature and migration of cells into areas of normal tissue are two common side effects of direct tissue transplantation or intravascular administration of MSCs (44-46). Additionally, differentiation potential of MSCs into multiple mesenchymal lineages (i.e., osteocytes, chondrocytes, and adipose tissue) raises long-term safety concerns as their transplantation may lead to cardiac ossification and/or calcification.

In contrast to MSCs, exosomes are non-viable and would not differentiate into inappropriate cell lineage or form tumors. Therefore, administration of them as a cell-free approach in cardiac regeneration could mitigate many of the safety concerns and limitations associated with the transplantation of viable MSCs.

Besides, the efficiency of exosome uptake has been correlated to intracellular and microenvironmental acidity (47). This provides a mechanism for exosome homing to ischemic tissues whereby MSC exosomes are preferentially endocytosed by ischemic CMCS which have low intracellular pH (48).

Interestingly, exosomes bear tetraspanins CD9 and CD81 on their membrane, which respectively direct them to CMCS that express intercellular adhesion molecule 1 (ICAM-1), a ligand of integrins after myocardial injury (49), and to vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (50). Besides, Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events, could facilitate cellular uptake of exosomes by specific cell types.

The use of viable replicating cells as therapeutic agents carries the risk that the biological potency of the MSC-Ex Cargo | Function
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Enzymes of ATP-producing phase of glycolysis ATP-generating phase of glycolysis | Inhibition of cardiac cells apoptosis via increasing ATP generation (54)
CD73, an adenosine-generating extracellular enzyme | Activation of adenosine receptors and induce Akt/GSK-3β survival kinase signaling pathway (55)
Peroxiredoxins and glutathione S-transferases | Lowering oxidative stress (56)
miR-22 | Interaction MeCP2 (57)
miR-221 | Inhibition of cardiac cell apoptosis via targeting PUMA (59)
miR-19 | Inhibition of PTEN, an inhibitor of the AKT survival signaling pathway (59)
Unspecified factor(s) | Prevention of adverse cardiac remodeling (55)
Unspecified factor(s) | Prevention of inflammatory response (61)
Unspecified factor(s) | Neovascularization (43)

Figure 2. Therapeutic advantageous of MSCs secretion (exosomes) in cardiac regeneration. Exosomes derived from MSCs can suppress the immune response, inhibit apoptosis, enhance angiogenesis, activate cell proliferation, and prevent adverse cardiac remodeling.
agent may persist or be amplified over time when the need has been resolved, and cannot be attenuated after treatment is terminated (dose second draft)

Furthermore, despite being smaller than a cell, exosomes are relatively complex biological entities that contain a range of biological molecules, including proteins and RNA, making them an ideal therapeutic candidate to treat complex injuries such as MI/R injury. Moreover, exosomes are significantly stable in culture, allowing for their large quantity collection from a culture medium through their release over periods of time. Thus it is possible to develop scalable systems for exosome production. Packed-bed reactors and hollow-fiber bioreactors (HFBR) have been employed in biotechnology for years, and their utility in the efficient and scalable production of exosomes has recently been reported (51). They provide an extensive growth surface area supporting a large numbers of cells at high densities and allow the secreted exosomes to be collected from the reactor perfusate. In some applications collection of secreted products can be maintained over several months of continuous production.

Following their harvest or collection, by employing one or more distinct properties or characteristics of exosomes, including their size, density, morphology, composition, zeta potential, biochemical or immunological feature, these nano-sized particles are purified. Methods successfully employed include velocity and density gradients, agglomeration or precipitation via volume-excluding polymers, such as PEG or identified peptides, and a number of commercially available kits employing such proprietary means as active-ligand coated beads for adsorption chromatography (52). They can also be stored without potentially toxic cryopreservatives at -20°C for six months with no loss to their biochemical activity. In addition, much the same with conventional pharmaceutical products drug, exosomes can be tested in terms of dosage and biological activity.

However, demand for highly advanced purification and characterization technologies and significantly variable content of exosomes are two of the main obstacles in developing and utilization of exosomes in the context of regenerative medicine.

**Therapeutic effects of MSC-EXs in CVD**

**Anti-apoptotic effects**

Reduced ATP production, increased oxidative stress and apoptosis all are key features of cell death after myocardial ischemia/reperfusion (I/R) injury. Under this circumstance the lack of oxygen disrupts aerobic glycolysis and ATP production, leaving anaerobic glycolysis as the major source of ATP production. Reduced ATP production is a key feature of cardiac cell death during myocardial ischemic injury. As MSC-EXs were found to contain all five enzymes required for the ATP-generating phase of glycolysis, the investigators hypothesized that MSC-EX can increase ATP production in CMCs and attenuate their post-ischemic apoptosis. In line with this hypothesis, Lai et al. (53, 54) demonstrated that exosome treatment restored energy depletion, leading to limit cell death after myocardial I/R injury. Moreover, it was shown that MSC-EXs carry active CD73, an adenosine-generating extracellular enzyme, on their surface, which could activate adenosine receptors and induce Akt/GSK-3β survival kinase signaling pathway in CMCs (55, 56). Furthermore, peroxiredoxins and glutathione S-transferases, present in MSC-EXs, resulted in decrease of oxidative stress, a crucial cause of cardiac cell death. Altogether, available studies suggested that by inhibition of apoptosis via ameliorating ATP depletion and stimulation of survival pathways, MSC-EXs provide a short time window of opportunity for the cardiac cells to retrieve other molecular dysfunction leading to cardioprotection benefits.

A recent study conducted by Feng et al. (57) revealed that preconditioned murine BM-MSCs release exosomes enriched with miRNA-22 (miR-22). When CMCs were co-cultured with these MSCs, these released exosomes were internalized within the CMCs and delivered their miR-22 cargo. Subsequently, interaction of miR-22 with methyl CpG binding protein 2 (Mecp2), which reported to be elevated in cardiac abnormalities, resulted in protection of CMCs (58).

Another study carried out by Yu et al. (59), exhibited that exosomes derived from rat BM-MSCs transduced with GATA-4, contained high levels of several miRNAs, including miR-221 and miR-19a. These exosomes were able to reduce apoptosis of ischemic CMCs via miR-221-dependent inhibition of p53-upregulated modulator of apoptosis (PUMA), a subclass of the Bcl-2 protein family. Furthermore, miR-19a cargo inhibited phosphatase and tensin homolog (PTEN), an inhibitor of the AKT survival signaling pathway, that resulted in activation of this survival pathway (60).

**Anti-inflammatory effects**

Apoptosis of CMCs during ICDs activates an inflammatory response that serves to clean up the injury site from dead cells and stimulate the repair off damaged tissue. However, this response may go far beyond and even extend the injury. Thus inhibition of inflammation response is considered as a therapeutic approach for this condition. MSC-EXs are known to participate in inhibition of inflammatory response (61). In their study Lee et al (61) reported that the MSC-EXs inhibits lung inflammation in hypoxic pulmonary hypertension (HPH), leading to relieving symptoms and reversing HP. In the context of ICD, administration of MSC-EXs in ischemic/reperfused myocardium led to reduction of local and systemic inflammation which accompanied by reduced infarct size and improved cardiac function (55). Thus, inhibition of inflammation reactions suggested being involved in protective effects against.

**Anti-cardiac remodeling**

Cardiac remodeling is a compensatory consequence of ICDs by which the remaining myocardium adapts to maintain cardiac function in the acute phase of injury. Remodeling encompasses cellular changes including CMCs hypertrophy, necrosis, apoptosis and fibrosis (62). In their study, Arslan et al. (55) indicated that the MSC-EXs are able to prevent adverse cardiac remodeling after myocardial I/R injury and MI via activating pro-survival signaling pathways, enhancing myocardial viability, restoring bioenergetics and reducing oxidative stress.
angiogenesis and cardiac repair. Exosomes (packaged nano-vesicles) have quickened to become a reality for therapeutic and paracrine effects of MSCs offers a rationale for employment of these cell-derived nanovesicles to replace viable cell transplantation. Exosomes can transport and deliver a large cargo of proteins, lipids, and nucleic acids and can modify cell and organ function. Much attention has been devoted to exploring the possible therapeutic roles of MSC-EXs in ICDs since they were discovered. Administration of these exosomes, which packaged with particular regenerative products, has shown promising cardiac regenerative and cardioprotective properties. Although a number of molecular mechanisms of exosomal-mediated cardiac repair are suggested, further investigation is required to fully understand the precise underlying mechanisms of these extremely encouraging nanovesicles. Future works will undoubtedly shed more light on the properties and functions of these natural carriers of biological molecules, paving the way for novel and exciting possibilities for the use of them in regenerative medicine including cardiac regeneration.

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MSC-derived exosomes in cardiac regeneration.

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