Cellular and Molecular Biology

Motavaf *et al. Cell. Mol. Biol.*2016, 62 (7): 74-79
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.7.13

Review

Therapeutic application of mesenchymal stem cell-derived exosomes: A promising cell-free therapeutic strategy in regenerative medicine

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Abstract: Mesenchymal stem cells have emerged as promising therapeutic candidates in regenerative medicine. The mechanisms underlying mesenchymal stem cells regenerative properties were initially attributed to their engraftment in injured tissues and their subsequent transdifferentiation to repair and replace damaged cells. However, studies in animal models and patients indicated that the low number of transplanted mesenchymal stem cells localize to the target tissue and transdifferentiate to appropriate cell lineage. Instead the regenerative potential of mesenchymal stem cells has been found - at least in part - to be mediated via their paracrine actions. Recently, a secreted group of vesicles, called "exosome" has been identified as major mediator of mesenchymal stem cells therapeutic efficacy. In this review, we will summarize the current literature on administration of exosomes released by mesenchymal stem cells in regenerative medicine and suggest how they could help to improve tissue regeneration following injury.

Key words: Mesenchymal stem cells, Exosome, Trans differentiation, Regenerative medicine.

Introduction

In recent years, regenerative medicine has emerged as a promising field to explore in tissue engineering and molecular biology. Underling process in this field deals with replacing, engineering, reprogramming or regenerating lost human cells or tissues to restore or maintain the normal organs' function. Currently, transplantation of stem cells has been suggested as the major approach in tissue regeneration. However, there is no solid evidence that cells employed to damaged target tissue, give rise to significant organ-specific cell populations through transdifferentiation. As a result, the concept of stem cell plasticity or transdifferentiation has been challenged recently (1).

There is mounting evidence that stem cells secrete a variety of soluble compounds (e.g. growth factors, cytokines, chemokines) and extracellular vesicles (EVs) that regulate their interactions with the surrounding microenvironment in a paracrine manner (2). The current data, related to paracrine effects of stem cells, are underpinned by the discovery of a controlled system to transport and deliver cellular cargos for which E. Rothman, R.W. Schekman and T. C. Südhof received the 2013 Nobel Prize for Medicine. This so called 'paracrine hypothesis' has gained much attention and is supported by several recent experimental observations. The paracrine hypothesis has inspired an alternative approach in regenerative medicine, which is based on using soluble factors and EVs secreted by the stem cells rather than the stem cells themselves. Accordingly, the regenerative function of stem cells has been suggested to be contributed to their paracrine effects- at least in part- rather than their actual transdifferentiation.

Available studies, related to use of these paracrine factors in tissue regeneration, have indicated promising results. These factors either released as soluble compounds or are transported in EVs. Their underlying regenerative effects is attributed to different mechanisms including inhibition of cellular apoptosis, stimulation of proliferation, reduction of oxidative stress, modulation of immune response, promotion of neovascularization and improvement of oxygen delivery.

EVs, including microvesicles, exosomes, ectosomes, and apoptotic bodies are small, spherical membrane fragments shed from the cell surface or released from the endosomal compartments. Microvesicles and exosomes have indicated to harbor pro-regenerative effects, which can be explained by the functional net result of their cargos including proteins, mRNAs, and regulatory non-coding RNAs (e.g. microRNAs). Exosomes are the most prominent type of extracellular vesicles. These nano-sized membrane vesicles of endocytic origin are secreted by most cell types including stem cells. They are released into the extracellular space upon fusion of multivesicular bodies (MVBs) with the plasma membrane and exert their diverse effects on target cells. The molecular mechanics for their secretion and uptake, as well as their composition, "cargo", and their resulting functions, are only beginning to be understood (3). As with other paracrine factors produced by stem cells,

Received February 27, 2016; **Accepted** June 9, 2016; **Published** June 30, 2016

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exosomes also provide a potential new approach in regenerative medicine.

MSCs-based therapy has been extensively investigated in the field of regenerative medicine for different organs (heart, kidney, lung, and liver) and showed beneficial results. However, as with other stem cells, cell-based therapy by MSCs raises several potential safety concerns. Increasing evidence demonstrated that MSCs also can synthesize and secrete bioactive soluble molecules and functional EVs including exosomes. Available data show that these beneficial effects can be attributed, at least in part, to secretion of these factors. In view of these findings, increasing body of researches have been conducted with the aim of investigating the regenerative properties of exosomes and introducing these "off the shelf" particles as a novel cell-free strategy in regenerative medicine.

Paracrine effect of MSC on regeneration

Currently, most studied approaches in regenerative medicine are based on transplantation of stem cells for regeneration of damaged organs. So far, various types of stem cells have been employed in several in vivo experiments and clinical trials (4). MSCs are the most widely used stem cells, as they are easily available in accessible tissues, such as bone marrow and adipose tissue (5), and they have enormous capacity for *ex-vivo* expansion. Furthermore, MSCs are known to have immunomodulatory functions (6) and therefore, could be applied in allogeneic transplantation. They are also reported to have highly plastic differentiation potential that included not only adipogenesis, osteogenesis and chondrogenesis (7), but also endothelial, cardiovascular (8), neurogenic (9) and neovascular differentiation.

The use of MSCs in clinical trials has increased exponentially in recent years with more than 400 trials listed in the ClinicalTrials.gov (10). The initial rationale for their clinical testing was based on their differentiation potential. However, the rare cell engraftment or transdifferentiate at the site of injury and the lack of solid experimental correlation between physical proximity of cell transplantation and functional improvement of target organ, have led to the proposal that MSCs exert their effects not through their transdifferentiation potential but through other alternative mechanisms (11-14). The current suggested mechanisms include cell fusion which leads to formation of heterokaryons (15, 16), cell-cell direct interactions and stimulation of resident stem cells differentiation (17), and paracrine effects (18).

Presently, the paracrine effect of MSCs is the most common proved mechanism. It was first described almost two decades ago when Haynesworth et al. (19) reported that MSCs synthesize and release a broad range of growth factors, chemokines and cytokines that could exert significant effects on cells in their proximity. Subsequent works indicated further paracrine functions mediated by EVs, including exosomes, released by MSCs. Recently, evaluating the regenerative potential of MSC-derived exosomes (MSC-EXs) have been begun to emerge.

Physical features and internal cargos of exosomes

Physical features

Exosomes were first characterized as organelles to remove cell debris or obsolete surface molecules out of the cell (20). However, in 1996 it was indicated that exosomes secreted by antigen-presenting cells (APCs), bear functional major histocompatibility complex (MHC) (21) suggesting that they are involved in antigen presentation, and immune communication.

During recent years, several studies have demonstrated that exosomes can be secreted by almost all cell lines and cell types. As shown in Fig. 1, the biogenesis of exosomes involves the tightly controlled process of inward budding from the membrane of MVBs. While some MVBs are degraded in lysosomes, exosome generating MVBs fuse with the plasma membrane and release their exosome content into biological fluids such as blood, urine, bile, saliva, CSF, and breast milk *in vivo*, or into culture medium *in vitro*. The number of exosomes present in biological fluids is extremely high, estimating to be about 3 million exosomes per microliter (22).

Exosomes are naturally produced, membrane vesicle-like structures that range in size between 30 and 100 nm in diameter. These small particles appear with a cup-shaped morphology when they are observed through transmission electron microscopy. Similar to all lipid vesicles, they float on sucrose gradients and their density ranges from 1.13 g/ml^{-1} (for B-cell-derived exosomes) (23) to 1.19 g/ml^{-1} (for intestinal cell-derived exosomes) (24). Thus, by flotation on sucrose gradients, they can be simply isolated from contaminating material, such as protein aggregates or nucleosomal fragments.

Composition of exosomes

Both ubiquitous and cell-specific membrane molecules (lipids and proteins) and loaded cargos (proteins, mRNAs, non-coding RNAs) are targeted selectively to exosomes. The former are more likely to be involved in exosome biogenesis, trafficking, structure and perhaps,

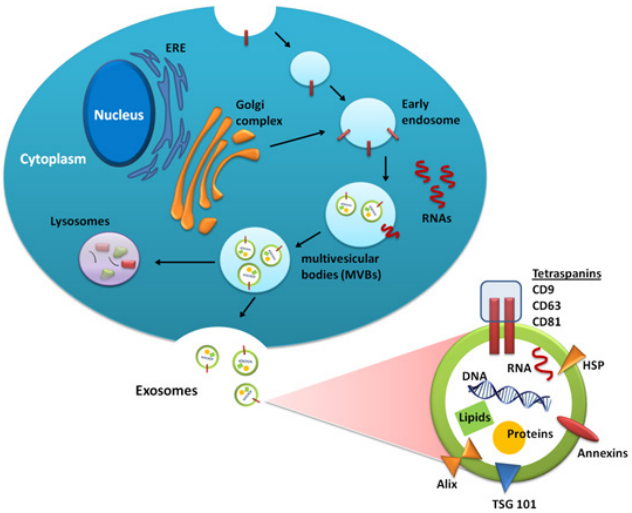

Figure 1. Biogenesis of exosomes. The formation of exosomes starts with an inward budding of the plasma membrane, leading to the formation of early endosomes. The incorporation of cytosolic protein/RNAs to endosomes leads to formation of multivesicular bodies (MVBs). The MVBs can fuse with the plasma membrane and release the exosomes to the extracellular environment.

in some unknown common functions. While cell-type specific components presumably, reflect the biological function of the parent cell mediated by secreted exosomes.mes provide a protective and controlled internal microenvironment, allowing their contents molecules to travel long distances within tissues without degradation. Evidence suggests that cargo packaging occurs non-randomly and that specific proteins, mRNAs and non-coding RNAs are preferentially sorted and shuttled into exosomes and secreted contents could vary in response to different stimuli (25-28).

So far only few studies have investigated the lipid composition of exosomes, with most of the lipid analytical work being performed on exosomes derived from cancer cells, reticulocytes, mast cells, B lymphocyte cells and human dendritic cells (DCs) (29, 30). It is indicated that exosomes are enriched in certain raft-associated lipids such as cholesterol, ceramide, sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains. These lipid rafts, function as sites for key membrane activities, such as endocytosis, cell adhesion and membrane trafficking. They have been reported in both cellular and exosomal membranes reflecting the endosomal origin of exosomes. Thus, they may represent a distinctive feature, which may be useful for their differentiation and isolation from other secreted microparticles. For example, phosphatidylserine, a lipid that is present normally at the cytosolic side of the plasma membrane, is also present at the surface of exosomes that are derived from platelets (31). The presence of lyso-bis-phosphatidic acid, a lipid that is enriched in late endocytic compartments, has been reported in B-cell-derived exosomes (32).

Ubiquitous proteins in exosomes include cytosolic proteins (such as tubulin, actin and actin-binding proteins) as well as membrane transport and intracellular membrane fusion proteins (annexins, flotillin and RAB proteins). Furthermore, molecules that are involved in signal transduction (such as protein kinases and heterotrimeric G proteins), proteins involved in multivesicle body biogenesis (Alix and TSG101) and constitutive isoforms of heat shock proteins (Hsp70, Hsp90) are found in exosomes. In addition, one of the most abundant protein families found in exosomes is tetraspanins. Several members of this family including CD9, CD63, and CD82 are highly enriched in exosomes from virtually any cell type (33, 34). These membrane proteins contain four transmembrane domains and are suggested to be involved in different biological functions of exosomes such as fusion, motility, immune modulation, adhesion, and cargo sorting.

Exosomes also contain proteins that are involved in specific cell functions. For instance, major MHC class II molecules are very abundant in exosomes from all cells that express MHC class II. By way of illustration, exosomes originated from DCs expressing MHCs are enriched with MHC II and express co-stimulatory molecules like CD54, CD80 and CD86 (also known as intercellular adhesion molecule-1 (ICAM-1), B7-1 and B7-2, respectively) suggesting a T-cell stimulatory capacity (35).

Proteins presented on the outer exoplasmic membrane layer of exosomes are routinely used as identifying markers. The most widely used markers are tetraspanins, Alix, flotillin, TSG101 and Rab5b, which are detected using antibody-based techniques (such as Western blot or ELISA) for the rapid confirmation of the presence of exosomes (36). Although protein transportation by exosomes was indicated almost three decades ago (37), in recent years a major breakthrough in exosome research was the finding of their nucleic acid contents such mRNAs, small non-coding microRNAs (miRNAs) and mitochondrial DNA (mtDNA) which can be transported to other cells (38). Since then, exosomes became more and more interesting in many research fields indicating a novel role as regulators in cell–cell communications during diverse biological processes (39, 40). At present, it is kwon that depending on desired net function, exosomes produced from a single cell can contain a heterogeneous population of compositions. This heterogeneity along with their stable physicochemical features, has led to the idea of using exosomes as natural nano-devices for the development of new therapeutic applications. A complete database of exosomal contents can be found at ExoCarta (www.exocarta.org).

Regenerative potential of MSC-EXs

Identification of MSCs secretion of exosomes has led to a growing body of studies on the therapeutic applications of these particles in regenerative medicine. Similar to other exosomes, MSC-EXs contain miRNAs, messenger RNAs and proteins, which can be transferred to recipient cells and subsequently exert their paracrine effects (41, 42). It is already known that, MSCs release exosomes that play a critical role in maintaining a microenvironmental niche for other cells. In addition to this function, it is postulated that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of other biochemical and cellular activities including tissue repairmen via promotion of regeneration. Thus, in recent studies the replacement of exosomes as a cell-free regenerative approach has gained much attention and the available data has conferred significant benefits in animal models of disease and tissue injury.

In late studies, the therapeutic potential of exosomes released from MSCs for the regenerative treatment of cardiac disease, especially acute myocardial infarction has been investigated. In their study, Zhao et al. (43) demonstrated that intravenous administration of human umbilical cord MSCs-derived exosomes, termed hucMSC-EXs, following acute myocardial ischemic (AMI) injury significantly increased cardiac function and reduced cardiac fibrosis. These significant therapeutic effects were indicated to be result of supportive effect of hucMSC-EXs which protected myocardial cells from apoptosis as well as promoted cell proliferation and angiogenesis. In other study conducted by Arslan et al. (44) MSC-EXs exerted their therapeutic effect on myocardial ischemia/reperfusion injury via increasing ATP levels, decreasing oxidative stress, as well as activating the PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after

In their study Xin et al. (45) evaluated the therapeutic effect of systemic administration of MSC-EXs on rat models of stork. They showed that the treatment

with these exosomes significantly improved functional recovery after stroke. In addition, this approach resulted in enhanced neurite remodeling, neurogenesis, and angiogenesis. In their other work, these authors has reported a significant increase in miRNA (miR-133b) content of exosomes released MSCs under the ischemic condition, which promotes neurological recovery from stroke (46). Based on these results, in their following work the authors (47) investigated the therapeutic effect of this approach on traumatic brain injury. This study also showed a significant improvement of traumatic brain injury via promoting endogenous angiogenesis and neurogenesis besides reducing inflammation in experimental rats. Even though, administration of MSC-EXs did not result in significant change in cortical lesion volume, but had other significant effects including improvement of cognitive and sensorimotor functional recovery, reduction of brain inflammation, increase in the number of newly formed neuroblasts and mature neurons in the dentate gyrus (DG), increase in the number of newly formed endothelial cells in the lesion boundary zone and DG.

Moreover, to examine the regenerative effects of hucMSC-EXs, Zhou and colleagues (48) used a rat model of acute kidney toxicity induced by Cisplatin. Following the treatment with Cisplatin and induction of acute kidney injury, the injection of hucMSC-EXs into the kidneys via the renal capsule, resulted in amelioration of acute kidney injury indices as well as promotion of rat renal tubular epithelial cells proliferation in culture. The results of this study also indicated that hucMSC-EXs have the ability to decrease apoptosis via reduction of Bax and increase of Bcl-2 levels and they can promote cell proliferation through stimulation of Erk1/2 pathway.

As it has been already indicated that MSCs can stimulate fibroblast migration, an essential phase of the wound healing process, without direct contact, Shabbir et al. (49) hypothesized that exosomes released from MSCs play a significant role in wound healing and regeneration of damaged tissue. The authors demonstrated that MSC-EXs promote AKT, STAT3, AKT and ERK signaling pathways by induction of expression of growth factors including hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1). The net result of these effects will promote cell proliferation and survival of target cells.

The regenerative effects of MSC-EXs were also evaluated on acute liver-injury both in vitro and in vivo. In a study conducted by Tan et al. (50) it was indicated that intraperitoneal injection of exosomes derived from human embryonic mesenchymal stem cell in mouse model of acute liver injury elicit hepato-protective effects. The regenerative effects of exosomes were suggested to be associated with induction of hepatocytes proliferation via upregulation of NF-κB, cyclin D1, and cyclin E as well as inhibition of their apoptosis, through upregulation of Bcl-xL.

Besides of their innate regenerative capability of exosomes released from MSCs, pre-treatment of exosomes cell source can manipulate the final therapeutic effects of these nanovesicles. For instance, overexpression of CXCR4 in MSC was indicated to result in production of CXCR4-enriched exosomes. CXCR4 is a critical factor involved in homing, endothelial cell migration, and engraftment of hematopoietic stem and progenitor cells. Cardiac treatment with these exosomes was indicated to protect cardiomyocytes from ischemic injury. Upregulation of the Akt signaling pathway contributed to these beneficial effects, suggesting that CXCR4-enriched exosomes may serve as an additional therapeutic strategy to promote cell survival and angiogenesis in ischemic hearts injury.

Regenerative advantages of exosomes over MSCs

Direct transplantation of MSCs into the target tissue or intravascular administration of them both are reported to result in multiple side effects including occlusion in the distal microvasculature and migration of these cells into areas of normal tissue (51-53). In addition, differentiation potential of MSCs into multiple mature cell lineages, including osteocytes and chondrocytes raises long-term safety concerns as their transplantation may lead to ossification and/or calcification.

In contrast to MSCs, exosomes are non-viable and will not differentiate into other inappropriate cell lineage or form tumors. Thus administration of them as a cell-free treatment in regenerative medicine could mitigate many of the safety concerns and limitations associated with the transplantation of viable cell. Furthermore, exosomes have been shown to be less immunogenic than actual cells, as a result of a lower content of membrane-bound proteins, such as MHC complex molecules (10). Thus, these nano-sized particles may readily been developed and applied with negligible alloimmunogenicity.

Other exciting feature of exosomes is that they are activated or attenuated in response to the release of injury-associated substrates, which in turn, is proportional to the severity of tissue damage and resolution of the injury. Thus, the efficacy of exosomes therapeutics effects could be highly responsive to as well all limited by the target tissue microenvironment. Besides, much the same with conventional pharmaceutical products drug, exosomes can be tested in terms of dosage and biological activity.

Another significant feature of exosomes is the encapsulation and protection of their cargo from degradation by enzymes or chemicals (54), which potentially prevents some of the problems associated with small soluble molecules such as cytokines, growth factors, transcription factors and RNAs, which are rapidly degraded in vivo .

Moreover, exosomes are significantly stable in culture, allowing for the collection of large quantities of them from a culture medium through their release over periods of time. Thus it is possible to develop scalable systems for exosome production. In addition, they can be stored without potentially toxic cryopreservatives at -20°C for six months with no loss to their biochemical activity.

Exosomes also bear several membrane proteins with binding affinity to ligands on target cell membranes or the extracellular matrix. These membrane bound molecules provide a potential mechanism for the directing and homing of exosomes to a specific tissue or microen-

vironment. For instance, integrins on exosomes could direct them to cardiomyocytes that express ICAM-1, a ligand of integrins after myocardial injury (55), or to (Vascular cell adhesion protein-1) VCAM-1 on endothelial cells (56).

Conclusion

Identifying exosomes as the central player mediating the therapeutic and paracrine effects of MSCs provides a rationale for shifting MSC-based therapy from a cellular to a non-cellular one. Encouraging therapeutic effects of MSC-EXs suggest that they hold great promise as new therapeutic strategy in regenerative medicine. Further characterization of MSC-EXs cargo and the mechanisms of their transfer and uptake by target cells are required to improve our knowledge in the field and to aid the translation of exosomes into clinical applications.

Acknowledgements
This work was supported by a grant from Tarbiat Modares University.

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