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The Use of Exosomes in Clinical Applications, With an In-Depth Look at Those Derived From Mesenchymal Stem Cells and Their Use in Myocardial Regeneration

Batla Al-Sowayan

King Abdullah International Medical Research Center/King Saud bin Abdulaziz University for Health Sciences, Stem Cell and Regenerative Medicine Department, King Abdul-Aziz Medical City - National Guard Health Affairs, Riyadh, Saudi Arabia

Abstract

Many mesenchymal stem cell (MSC)-based clinical trials are targeted toward cardiovascular diseases, specifically cardiac ischemia. Investigatory work has revealed that MSC-produced "exosomes" are in fact the element that initiates tissue restoration. This, in addition to the advantages of using exosomes over transplanting whole cells, makes "MSC-exosome-based therapy" a very appealing concept. This review will discuss exosomes and their possible clinical applications, with particular emphasis on MSC-derived exosomes and their use in managing ischemic myocardial damage.

***Keyword** - Mesenchymal stem cell, Exosomes, Myocardial repair, Ischemia, Myocardial infarction.*

1. Introduction

Cardiovascular diseases (CVDs) are a major cause of mortality and morbidity worldwide. Coronary artery diseases (CAD), or ischemic heart diseases (IHD), are the most common CVDs.[1] CAD/IHD occur due to buildup of plaque inside the arteries that supply blood to the myocardium. The plaque buildup causes these arteries to become hard and narrow; this condition is called atherosclerosis.[2] As atherosclerosis progresses, less blood can flow through the arteries. Consequently, the myocardium cannot get the nutrition and oxygen that are required for normal function. This can lead to angina or myocardial infarction (MI), commonly known as "heart attack". Progressive myocardial dysfunction following MI accounts for the most number of deaths as a result of CVDs.[3]

When the blood supply to the heart is disrupted, an ischemic cascade occurs. Ischemia, or cell starvation due to lack of oxygen, causes myocardial cells to die. A collagen scar will be formed to replace the lost cells, which leads to permanent damage to the myocardium.[4] Therefore, the main strategy to manage the onset of MI is to save myocardial tissue from further damage. Following a MI episode, the patient is often given a collection of drugs to initiate reperfusion and reduce physical exertion on the heart, thus protecting the ischemic myocardium.[5]

Effective drug therapy is an important research direction for limiting and controlling acute cardiac manifestations. However, recent advances in cellular therapy and regenerative medicine have prompted researchers to also explore the possibility of regenerating the damaged myocardial tissue following hypoxia to prevent future complications. Recently, we have witnessed major research progress in the use of mesenchymal stem cells (MSCs), a non-haematopoietic stromal cells, as a therapeutic agent for chronically injured organs. The ability of MSCs to differentiate into mesoderm and non-mesoderm derived tissues, their abilities in maintaining and replacing endogenous stem cell niches, their immunomodulatory properties, and their accessibility have made them one of the most extensively examined and clinically tested types of stem cell.[6]

Preclinical and early phase clinical trials showed that the administration of MSCs following MI reduced the cardiac complications and improved the clinical status of tested subjects.[7-10] However, an increasing body of evidence suggests that the means through which administered MSCs exert their tissue healing effect is by releasing paracrine factors. The paracrine hypothesis proposes that cells release soluble mediators that induce angiogenesis and have cardio-protective effects.[11,12] The chief paracrine factor being implicated is the exosome. In this review, exosomes and their possible clinical applications will be discussed.

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Then, studies where MSC-derived exosomes were used to manage myocardial damage following MI will be examined.

2. What are exosomes?

Cells release spherical particles called vesicles that are enclosed by phospholipid bilayers. The discovery of cell-derived vesicles dates back to the 1940s.[13] The recognition of exosomes as a distinct type of cell-derived vesicle, along with the term "exosome", emerged during the 1980s.[14] Exosome release was proposed to be the mechanism through which reticulocytes remove unwanted plasma membrane proteins before becoming erythrocytes.[15-17] Nowadays, it is well established that almost all mammalian cells produce exosomes that carry out numerous biological functions, depending on their cell of origin. The most important exosome function is facilitating intercellular communication. For example, exosomes can transfer antigens and transport receptors between immune cells to initiate modulation of the immune system.[18] In the nervous system, exosomes transmit neurosignals among neurons.[19] Exosomes can also transfer genetic information between different types of cell by transporting genetic materials.[20]

According to the International Society for Extracellular Vesicles, extracellular vesicles (EVs) are classified into four subtypes: exosomes, ectosomes, microvesicle particles, and apoptotic bodies.[21] The distinctions between these four EVs depend on their subcellular origin, composition, size, morphology, and density.

Subcellular origin. Exosomes are of endosomal origin. Within the cell, cell content is sorted via membrane bound compartments called endosomes. First, endocytic vesicles, formed by invagination of the plasma membrane, fuse with early endosomes and release their proteins/lipids content. Content intended for recycling is placed into a recycling endosome.[22] Then, the early endosome will become a late endosome or multivesicular body (MVB).[23] Content of the MVB will be packed into small vesicles called intraluminal vesicles (ILVs).[23] Depending on their content, ILVs are sent for either degradation or exocytosis. ILVs released into the extracellular space are called exosomes.[24,25] The constituents of the exosomal cargo and its lipid encasement depend on the cell of origin, its state and its microenvironment.[26] They are mainly proteins, lipids, messenger RNAs (mRNAs) and micro RNAs (miRNAs).[27]

Composition. Exosomes are enriched in an a set of proteins that can be used for characterization purposes. Mathivanan and Simpson have created "ExoCarta", an exosome database (www.exocarta.org)[28] that provides a list of the most commonly identified proteins in exosomes based on submission of independent exosome examinations. Researchers often pick one or more of these proteins as markers for exosome characterization. For example, in a study of human bone marrow MSC (BMMSC)-derived exosomes, purified exosomes where detected by using CD9 and CD81 as protein markers, in addition to shape and size assessment.[29] Exosomes may also contain distinguishing proteins that reflect their cellular origin. For example, unlike other exosomes, immune cell-derived exosomes are enriched in major histocompatibility complex II protein.[30] In maternal circulation, placenta-derived exosomes can be distinguished by the presence of placental alkaline phosphate protein.[31]

Size and morphology. Exosomes have round, cup-shaped morphology and are generally found to be 50 to 100 nm in diameter by transmission electron microscopy.[32] However, some researchers accept a wider range that goes down to 30 nm.[33,34] It is very important to note that the diameter of an exosome is largely dependent on the technique used to measure it. For example, the diameter of MSC-derived exosomes was around 30-50 nm when measured by scanning electron microscope; when the same sample was measured using dynamic light scattering, the diameter was 208 nm; nanoparticle tracking analysis indicated a diameter of 110 nm.[35]

Density. The most widely used exosome harvesting protocols are based on a characteristic density of 1.13–1.19 g/ml in sucrose. The protocols include differential centrifugation and/or filtration to remove dead cells and debris, followed by ultracentrifugation coupled with a sucrose density gradient.[36,37] However, the sucrose density gradient method is quite laborious and time consuming. Hence, other exosome harvesting techniques are being developed based on different principles. One such method uses high-performance liquid chromatography, where the conditioned medium "mobile phase" passes through a column (the "stationary phase"). In the column, the constituents of the medium travel at different speeds, allowing the separation and collection of exosomes.[35,38] Immune-based isolation protocols are also being established, in which exosomes are collected via capture antibodies.[30] Whereas most commercial kits now being developed for exosome isolation use a precipitation-based principle.[39,40]

3. Clinical perspective on exosome-based therapy and its advantages

Regardless of the application, there are general advantages in using exosomes over the "whole-cell approach". First, exosomes are highly tolerated by the immune system. This allows repeated administration of therapeutic exosomes without the risk of immune activation or the need for immunosuppressive drugs.[30] Second, delivering exosomes to target tissues is easier than delivering whole cells[41]; membrane bound proteins can be modified to allow exosome uptake by a specific cell type.[42] Third, these

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extracellular organelles are not viable, which means that they do not replicate, hence there is no risk of tumor formation.[43] Fourth, exosomes can cross the blood-brain barrier (BBB)[44]; this is of great value when the central nervous tissue is targeted.

As a source of exosomes, MSCs in particular, are very practical. MSCs have been clinically tested and safely administered in hundreds of clinical trials. This indicates that MSC-derived exosomes should be safe to administer as well. MSCs can be isolated from several body tissues, including tissues that are considered medical waste such as placenta.[45] Also, MSCs can be expanded in vitro on a large scale; such availability of parent cells allows mass production of exosomes. In addition, MSC-derived exosomes were shown to have an intrinsic tissue homing potential[46], and intrinsic tissue healing properties.[47, 48] All these traits make MSC-derived exosomes ideal for development into off-the-shelf therapies. Currently, exosomes are being explored in four main applications:

3.1. As a carrier

Some therapeutic agents require "a vehicle" to deliver them to their target. This could be because: the bioactivity of a given agent will be lost if administered into the body; or the target is intracellular and the agent is not capable of crossing the cell membrane; or the target is present in several cell types and if administered without an enclosing vehicle the agent will be taken up by non-diseased cells and lead to adverse effects.[49] Exosomes, are being examined as a vehicle to carry and deliver therapeutic molecules to specific tissue targets. Exosomes carry out most of their normal functions by taking cargo from one cell and off-loading it into another. Exosomes could be loaded with therapeutic proteins and peptides, miRNAs or drugs.[46] They are ideal vehicles for molecule delivery, because they can carry a relatively large cargo and guard it against degradation. Their bilipid membrane is highly efficient in protecting the cargo, giving a long shelf-life and half-life.[46]

As a carrier, exosomes are mostly employed to deliver antitumor miRNAs; miRNAs are small, noncoding RNAs that bind to mRNA and regulate gene expression, including tumor suppressors.[50] Antitumor miRNA therapy is developing as a promising anticancer intervention. However, in vivo application of miRNA-based therapy has been limited by the lack of an efficient delivery technique.[51] There are a quite a few successful in vivo studies that demonstrate the feasibility of the exosome-miRNA carrier approach. Katakowski et al. (2013) used MSC-derived exosomes in a rat brain tumor model. MSCs were first transfected with the antitumor miRNA miR-146b. Then, exosomes from the transfected cells were harvested and injected directly into the tumor; this led to a significant reduction in glioma growth. Exosomes are also being investigated as drug carriers. In one study, exosomes were loaded with anti-inflammatory drugs, and were administered nasally to mouse brains. Three different inflammation-mediated brain disease models were investigated: a lipopolysaccharide-induced brain inflammation model, an implanted brain tumor model, and experimental autoimmune encephalitis. In all three, exosomes were able to cross the BBB and deliver and unload the drug in the targeted tissue, which in turn reduced brain inflammation[52]

3.2. Modulating immune cell signaling

In physiological conditions, exosomes can suppress immune responses. It is believed that in the placenta, trophoblasts release exosomes that carry the immunosuppressive molecule HLA-G. This plays an important role in fetus tolerance during the first trimester.[23] In the immune system, activated T cells produce FasL-carrying exosomes that prompt induced-T cell death.[53] Moreover, it was demonstrated that trophoblast-derived exosomes can confer viral resistance to other cells by delivering the placenta-specific miRNA C19MC.[54] For MSC-derived exosomes, it was reported that human BMMSC-derived exosomes promote tumor growth in an animal model. MSC-derived exosomes increased tumor cell expression of vascular endothelial growth factor, which in turn increased angiogenesis and therefore tumor growth.[29]

There are contradictory reports on MSC administration and their immunomodulatory effects on human cancers. Waterman et. al. (2010) reported that MSCs can have two phenotypes, a proinflammatory "MSC1" and an immunosuppressive "MSC2" phenotype. MSCs express several toll-like receptor (TLRs), including TLR3 and TLR4.[55-57] When MSCs were primed with TRL4, they increased the secretion of proinflammatory factors, whereas TLR3 priming caused MSCs to increase the secretion of factors with immunosuppressive properties.[55,56] In an in vitro study, co-culture of MSC1 with cancer cell lines attenuated cancer cell growth, migration and invasion, while MSC2 co-culture with cancer cells had the opposite effects.[58] However, in an in vivo study using a diabetic peripheral neuropathy (pDPN) mouse model, administration of BMMSC2s significantly improved the symptoms of pDPN compared to non-primed BMMSC treatment.[59] This improvement was most likely due to changes in the immune modulating factors found in the serum of MSC2-treated mice.[59].

A cell can release exosomes that exert different actions depending on the phase of cell maturation.[34] For example, exosomes released by immature dendritic cells are tolerogenic, whereas exosomes released by mature dendritic cells are immunogenic.[60] Hence, it is possible that MSC-derived exosomes might also be proinflammatory or immunosuppressive depending on the MSC phenotype, i.e.

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MSC1 or MSC2, they were derived from. Therefore, to obtain the desired immune-modulating functions, a choice should be made between MSC1-derived exosomes and MSC2-derived exosomes in MSC-exosome-based therapy.

3.3. For tissue repair

Mechanism of action studies revealed that administered cells exert tissue healing effects mainly through the release of paracrine factors including exosomes. Hence, because of the advantages of exosome-based therapy over whole cell-based therapy, the ability of exosomes, by themselves, to initiate tissue repair is being investigated. The regenerative ability of MSC-derived exosomes was first tested in an animal model of myocardial ischemia/reperfusion (MI/R) injury. The study and follow-up studies revealed that exosomes are the key player in MSC-based myocardial repair. These findings and other investigatory work on myocardial repair by MSC-derived exosomes are discussed further in section 4. The therapeutic potential of MSC-derived exosomes for tissue repair was then expanded to include other tissues such as kidney[47], liver[61] and brain. Central nervous system tissues are a major target in exosome-based regenerative therapy because of the ability of exosomes to cross the BBB. In two studies conducted by the same group, Xin et al. (2012) reported that in a middle cerebral artery occlusion (MCAo) rat model, miR-133b is down regulated. Following MSC administration, miR-133b was significantly increased in brain tissue. It was proposed that the miR-133b increase in neurons was the result of MSC release of exosomes that carried the therapeutic miRNA to affected cells, which in turn improved functional recovery.[37] To confirm this hypothesis, Xin et al. (2013) administered cell-free exosomes generated from MSCs to the same MCAo rat model. Improved functional recovery was again observed in treated rats, and again it was proposed to be due to miRNA contained within the exosomes.[62] Increased angiogenesis, neurogenesis and functional recovery were also observed in a traumatic brain injury rat model following MSC-exosome administration by the same group.[40]

3.4. As a diagnostic tool

Cells modify their exosome cargo in response to injury; these modifications can be used as a diagnostic tool.[63] For example, in the case of malignancies such as melanoma[64] and ovarian cancer[65], malignant cells release exosomes with distinct miRNAs from the miRNAs in nonmalignant-cell-derived exosomes. In cancer patients, these exosomes could be used as biomarkers of tumor development. It was also proposed that measuring the level of placenta-derived exosomes in maternal circulation could be used as a biomarker for pre-eclampsia.[66] For cardiovascular diseases, patients with an injured myocardium have exosomes that contain high levels of miR-133a in their circulation[67]; again, this could be used as a biomarker of disease development.

4. MSC-derived exosomes for myocardial repair

It was first suggested by Gencchi et al. (2005) that the functional improvement following 72 h of BMMSC administration in an animal MI model could not be attributed to cell regeneration by transplanted cells, but was instead achieved through paracrine factors released by these cells in situ. Gencchi et al. then supported this hypothesis by demonstrating that conditioned medium from rat BMMSCs alone was enough to exert cardio-protective effects both in vitro and in vivo.[68,69] Timmers et al. (2007) then reported that conditioned medium from human embryonic MSCs (hEMSCs) reduced infarction size by 60% in a MI/R animal model. Study of the conditioned medium revealed that the paracrine factor responsible for the cardio-protective effect was a fraction of the conditioned medium that was 100-220 nm in diameter.[70] Lai et al. (2010) also administered hEMSC conditioned medium to MI/R animal models, and noted a significant reduction in infarction size. The cardio-protective complex in the medium was hypothesized to be exosomes. Indeed, when Lai et al., administered isolated exosomes from the conditioned medium, similar results were observed.[71] Later study confirmed this hypothesis; in an ischemia/reperfusion animal model, it was reported that a single intravenous administration of hEMSC-derived exosomes was enough to reduce infarct size by 45%.[38] In another study, reduced infarct size was also seen in an acute MI animal model following rat BMMSC-derived exosome administration. Moreover, it was observed that there was no difference in outcomes between the exosome-depleted conditioned medium-treated group and the control group.[72] This suggests that other paracrine factors in the medium have a negligible effect in initiating repair.

The most postulated mechanism of action for exosomes is through the transfer of mRNAs, miRNAs and proteins. miRNAs are particularly being associated with an increasing number of exosome-mediated actions. miRNAs can regulate a wide-range of biological processes by binding to target mRNA. A miRNA can lead to translational repression or target degradation of the specific mRNA.[33] Barile et al. (2014) reported that cardiac progenitor cell (CPC)-derived exosomes were rich in miR-210. miR-210 down regulates ephrin A3 and PTP1b, which in turn inhibits cardiomyocyte apoptosis. CPC-derived exosomes were also rich in miR-132, which stimulates tube formation by down regulating its target RasGAP-p120.[73] In another study, where CPC-exosomes also inhibited cardiomyocyte apoptosis, analysis of the exosomes revealed high levels of miR-451, which is a GATA4-responsive miRNA (36). GATA4 is a critical transcription factor that is essential for cardiac development.[74] For MSC-derived exosomes, Arslan et al. (2013) proposed that some of the >800 proteins found in MSC-derived exosomes must have activated cardio-protective pathways. These pathways include decreasing the

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oxidative stress, increasing ATP production and P13K/Akt signaling. Feng et al. (2014) reported that exosomes produced by hypoxia-exposed MSCs are more efficient in reducing cardiac fibrosis in an animal MI model, because they are enriched in miR-22.[75] Yu et al. (2015) reported that exosomes produced by MSCs overexpressing GATA4 had higher miR-19a levels, and thus were more efficient in initiating recovery of a myocardial ischemia/infarction animal model.[76]

5. Final remarks

Ischemic heart diseases that cause dysfunction or loss of cardiomyocytes (cardiac muscle cells, the main constituent of the heart) are a leading cause of death worldwide.[77] Hence, cardiac ischemia, and cardiovascular diseases in general, are major targets for many clinical trials including MSC-based trials. The trend now, however, is shifting toward using the MSC product "exosomes" as an alternative. This shift is due to the advantages of using exosomes over transplanting whole cells, and because of recent findings that the exosomes are in fact the element that initiates tissue restoration.

A growing number of studies are consistently presenting exosomes as the most vital paracrine mediator to initiate tissue repair in a cell-independent manner. Exosomes alone, as discussed earlier, can successfully regenerate various tissue types, including cardiac muscle tissue. Recent findings have also revealed that, like cells, exosomes can be "customized" to become more effective in specific pathologies. Preconditioning or genetic engineering of parent cells can prompt the production of "disease-specific exosomes" that are more effective in targeting a given condition. This will be very effective, not to mention very practical, in clinical settings for the rapid protection and restoration of cardiomyocytes following ischemia.

Nevertheless, these extremely promising findings, as expected, come with challenges. The main challenge is quality control in exosome production. There is no standardized method for cell preparation and exosome collection and purification. In all methods used currently, producing a consistent yield of exosomes that are sterile for in vivo application is still a work in progress. Another issue is exosome dosage and delivery. It was reported that no exosomes were found in the liver of an animal model following intranasal administration of drug-loaded exosomes[52], whereas exosome accumulation occurred in the liver 24 h after mouse tail vein injection.[51] Hence, it is of extreme importance that exosomes are administered in a manner that protects them from the normal bodily clearing mechanisms so that they reach their target and achieve maximum outcomes.

Exosomes can be harvested from almost any cell type; however, MSC-derived exosomes could be a very practical exosome subtype. MSCs can be isolated in large quantities from various tissue sources, which facilitates initial cell harvesting. MSCs are also relatively easy to expand in the laboratory, allowing exosome isolation on a large-scale. Having such access to exosome-producing cells will enable large-scale exosome production. Moreover, MSCs have been extensively studied and shown to be safe to administer in clinical settings. Recent studies have shown that MSC-derived exosomes are also safe to administer, and, like the parent cells, have intrinsic homing and tissue healing abilities. All these factors favor the use of MSC-derived exosomes in *in vitro* and *in vivo* studies. Furthermore, they make MSC-exosomes ideal for development into ready-to-use products for clinical applications.

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