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Therapeutic potential of mesenchymal stem cell-derived microvesicles

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Abstract

Several studies have demonstrated that mesenchymal stem cells have the capacity to reverse acute and chronic kidney injury in different experimental models by paracrine mechanisms. This paracrine action may be accounted for, at least in part, by microvesicles (MVs) released from mesenchymal stem cells, resulting in a horizontal transfer of mRNA, microRNA and proteins. MVs, released as exosomes from the endosomal compartment, or as shedding vesicles from the cell surface, are now recognized as being an integral component of the intercellular microenvironment. By acting as vehicles for information transfer, MVs play a pivotal role in cell-to-cell communication. This exchange of information between the injured cells and stem cells has the potential to be bi-directional. Thus, MVs may either transfer transcripts from injured cells to stem cells, resulting in reprogramming of their phenotype to acquire specific features of the tissue, or conversely, transcripts could be transferred from stem cells to injured cells, restraining tissue injury and inducing cell cycle re-entry of resident cells, leading to tissue self-repair. Upon administration with a therapeutic regimen, MVs mimic the effect of mesenchymal stem cells in various experimental models by inhibiting apoptosis and stimulating cell proliferation. In this review, we discuss whether MVs released from mesenchymal stem cells have the potential to be exploited in novel therapeutic approaches in regenerative medicine to repair damaged tissues, as an alternative to stem cell-based therapy.

Keywords: exosomes; kidney injury; mesenchymal stem cells; microvesicles

Introduction

Mesenchymal stem cells, also known as multipotent mesenchymal stromal cells (MSCs), have been the focus of great interest in regenerative medicine for their ability to migrate to the site of injury, as well as for their multilineage differentiation potential and their straightforward *in vitro* expansion.

For their potential therapeutic application in acute tissue injury of different organs (heart, kidney, lung and liver), MSCs are currently used in clinical trials for treating a wide range of diseases (http://www.clinicaltrials.gov). Recent studies have however suggested that the beneficial effect of MSCs in cells of injured tissues is not attributed to their differentiation, but rather to the activation of a protective mechanism and stimulation of endogenous regeneration. This contention is supported by the production of bioactive soluble factors known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-intrinsic progenitor cells [1] and modulate the immune response [2].

MSC-secreted bioactive molecules may act as paracrine or endocrine mediators that directly activate target cells and/or cause neighbouring cells to secrete functionally active agents [1]. It has recently been demonstrated that extracellular vesicles or microvesicles (MVs) released from cells are an integral component of the cell-to-cell communication network involved in tissue regeneration [3, 4], and therefore may contribute to the paracrine action of MSCs.

Paracrine/endocrine mediators of MSC regenerative action

The role of bone marrow-derived MSCs in the recovery of acute kidney injury (AKI) has been extensively studied. MSCs were shown to accelerate recovery from AKI induced by toxic agents [5, 6] or ischaemia/reperfusion [7], as well as to induce functional improvement in chronic kidney disease [8]. Despite early accumulation of systemically administered MSCs at the site of injury, few MSCs permanently engrafted within the kidney [5–7] and, at least in the model of glycerol-induced AKI, the majority of MSCs were no longer present after a few days [9]. It has therefore been suggested that MSCs do not act by replacing renal tubular cells, but alleviate injury by providing a paracrine support to the repair. Humphreys *et al*. [10], using genetic fate-mapping techniques, demonstrated that repopulation of the tubules following AKI is performed by surviving uninjured tubular cells. Further support for the paracrine/endocrine action of MSCs is provided by experiments showing that MSC-conditioned medium (CM) mimics the beneficial effects of the cells of origin [11]. This study proves definitively that homing is not an absolute requirement for MSC-based therapy, as

the intraperitoneal administration of CM to mice with cisplatin-induced AKI was sufficient to limit renal injury [11]. It can be therefore concluded that the renoprotective effect of MSCs depends on secreted factors. Among these factors, insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF) have been shown to play relevant roles as IGF-1 gene silencing [12] and VEGF knockdown [13] limited the protective effect of MSCs on renal function and tubular repair.

Studies demonstrating the protective role of MSCs have also been reported in other organs, including the liver, lungs and heart. In preclinical animal models of myocardial infarction, MSC treatment improves perfusion, reduces myocardial scarring and restores cardiac function. Gnecchi *et al*. [14] showed that CM from MSCs overexpressing the Akt gene (Akt-MSCs) reduced infarct size in a rodent model of acute myocardial infarction as efficiently as the actual MSCs. This cardioprotective effect of the CM was subsequently attributed to the Akt-MSC-secreted frizzled-related protein 2 (Sfrp2) [15]. Recently, the extracardiac administration of MSCs, in order to target the skeletal muscle, provided clear evidence that cardiac repair can be achieved through MSC trophic actions, independent of the myocardium localization of stem cells [16]. In addition, in a rat model of global heart failure, MSCs were able to attenuate myocardial fibrosis by the secretion of the anti-fibrotic factor, adrenomedullin (ADM) [17].

Another example of the paracrine action of MSCs is demonstrated in their immunomodulatory properties. MSCs can inhibit several T-lymphocyte activities [18] and are able to alter the cytokine production of dendritic cells (DCs), naïve and effector T cells and natural killer cells (NK), resulting in a more tolerant/anti-inflammatory phenotype. Moreover, MSCs stimulate the production of regulatory T (Treg) cells, leading to down-regulation of the immune response. MSCs constitutively express COX-2 and synthesize prostaglandin E2 (PGE2) that partly accounts for the immunomodulatory action on T lymphocytes [18]. The inhibitory role of MSCs on NK functions can be attributed to the secretion of indoleamine 2,3-deoxygenase, PGE2 and transforming growth factor-β1 (TGF-β1) [18]. In addition, MSCs secrete interleukin-6 (IL-6) promoting the reversion of maturation of DCs, resulting in a less mature phenotype [19]. The generation and expansion of Treg cells from $CD4^+CD25^-$ precursors is stimulated by TGF-β1 and PGE2. Moreover, MSCs also secrete the soluble MHC isoform of human leucocyte antigen G5 (HLA-G), contributing to the expansion of $CD4^+CD25highFOXP3^+$ Treg cells [20].

Role of extracellular vesicles released from MSCs as paracrine/endocrine mediators

Extracellular vesicles have been recently considered as important mediators of cell-to-cell communication. These vesicles can be categorized into exosomes and shedding vesicles. Exosomes arise from the endosomal membrane cell compartment and are released into the extracellular space after fusion of multivesicular bodies with the plasma membrane [21–23]. Exosomes, tend to be homogenous in size (30–120 nm) and are released by p53-regulated exocytosis, which is dependent on cytoskeleton activation but independent of cell calcium influx. Exosomes are rich in annexins, tetraspanins (CD63, CD81, CD9) and heat-shock proteins (such as Hsp60, Hsp70 and Hsp90), expose low amounts of phosphatidylserine and include cell-type-specific proteins. In addition, tumour susceptibility gene 101 (Tsg101), Alix and clathrin are frequently present in exosomes. Shedding vesicles, also known as ectosomes or microparticles, originate from direct budding and blebbing of the plasma membrane of many different cell types [21–23]. Shedding vesicles are more heterogeneous in size, ranging from 100 nm to 1 μm, and are released by budding of small cytoplasmic protrusions, which is dependent on calpain, cytoskeleton reorganization and intracellular calcium concentration. Calcium ions are responsible for the changes in asymmetric phospholipid distribution of the plasma membrane that lead to the formation of shedding vesicles. These vesicles expose high amounts of phosphatidylserine, contain proteins associated with lipid rafts and are enriched in cholesterol, sphingomyelin and ceramide [21, 22]. As both exosomes and shedding vesicles are present *in vitro* and *in vivo*, this mixed population is collectively known as MVs. MVs contain surface receptors, biologically active molecules such as proteins and lipids, as well as mRNA and microRNA. There is another type of extracellular vesicles, larger than 1 μm, which is the apoptotic body, derived from dying cells. Inside these vesicles, DNA is frequently present as a residue of the nucleus.

The distinctive features of the three types of extracellular vesicles are summarized in Table 1.

MVs may interrelate with target cells by specific receptor–ligand interactions and transfer receptors and biological active molecules to these target cells following internalization [4]. Moreover, besides proteins and lipids, MVs may also carry mRNA and microRNA and thus could play a role in the exchange of genetic material between cells (Figure 1) [3, 24–26].

MV biological activities

MVs may influence the behaviour of recipient cells by several different mechanisms. First, they may act as signalling complexes by direct stimulation of target cells. Indeed, MVs express several types of receptors and surface molecules, including: tissue factor (TF), tumour necrosis factor (TNF), MHC Class I/II molecules and CCR5 chemokine receptor [22, 23, 27]. This results in MV-mediated activation of cells bearing specific ligands for these receptors. For example, MVs expressing the intercellular adhesion molecule 1 (ICAM1) at their surface can interact with the lymphocyte function-associated antigen 1 (LFA1), a ligand for ICAM1, present on the membrane of CD8+ DCs, thus activating T cells [28]. MVs expressing the delta-like 4 (Dll4), a transmembrane Notch ligand, may activate angiogenesis and axon growth by interacting with Notch receptors expressed by endothelial or nerve cells, respectively [29].

Table 1. Characteristics of exosomes, shedding vesicles and apoptotic bodies

	Exosomes	Shedding vesicles	Apoptotic bodies
Size (nm)	30–120	100–1000	≥1000
Biogenesis	By exocytosis of multivesicular bodies Process dependent on cytoskeleton activation and Ca^{2+} independent	By budding of plasma membranes. Process dependent on Ca^{2+}, calpain and cytoskeleton reorganization	By blebbing of plasma membranes of dying cells
Markers	CD63, CD81, CD9, Tsg101, Alix, Hsc70 Low exposure of PS Markers specific to the cell of origin, e.g. PECAM in platelet vesicles and EGFRvIII in vesicles from gliomas	Lipid raft-associated molecules (TF, flotillin) High exposure of PS	Exposure of PS
Content	Proteins, lipids, mRNA and microRNA, rarely DNA	Proteins, lipids, mRNA and microRNA, rarely DNA	Fragmented DNA

EGFRvIII, epidermal growth factor receptor variant III; Hsc70, heat-shock cognate protein 70; PECAM, platelet endothelial cell adhesion molecule; PS, phosphatidylserine; TF, tissue factor; Tsg101, tumor susceptibility gene 101.

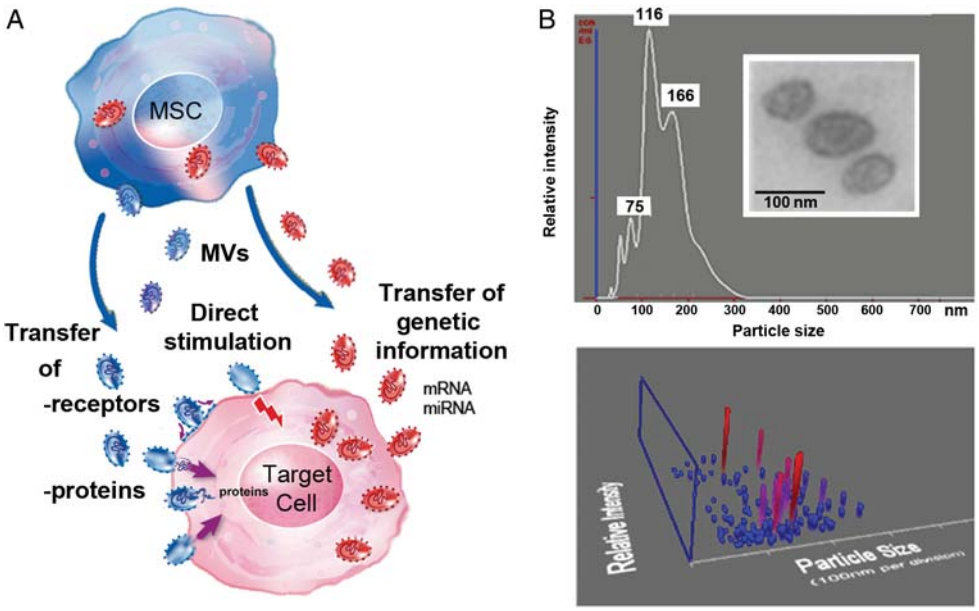

Fig. 1. MVs released from MSCs. (**A**) Schematic representation of MV-mediated intercellular communication. MVs may directly stimulate target cells through surface-expressed receptors. MVs may transfer receptors or proteins from the cell of origin to the target cell. MVs may convey genetic information by horizontal transfer of mRNA and microRNA (miRNA) inducing functional changes in the target cell. (**B**) Nanosight analysis of MVs purified from MSCs. The mean size and particle concentration values are calculated by the Nanoparticle Tracking Analysis software that allows the analysis of video images of the particle movement under Brownian motion captured by Nanosight LM10 and the calculation of diffusion coefficient, sphere equivalent and hydrodynamic radius of particles by using the Strokes–Einstein equation. The curve describes the relationship between particle number distribution (left *Y*-axis) and particle size (*X*-axis). The inset shows MVs seen by transmission electron microscopy.

MVs may also transfer receptors and/or bioactive lipids between cells after fusion with the target cell membrane. For example, the chemokine receptor CCR5 or CXCR4 [30] could be transferred from T cells to non-lymphoid cells, rendering them susceptible to HIV infection [27, 30, 31].

After ligand interaction, MVs may also modulate the functional target cell by delivering intracellular proteins. MVs derived from endothelial cells can activate angiogenesis through the transfer of pro-angiogenic molecules such as growth factors (VEGF, basic fibroblast growth factor bFGF, platelet-derived growth factor PDGF, leptin, acidic fibroblast growth factor aFGF, TNFα, and TGF-β and others) [32], along with proteases (e.g. matrix metalloproteases MMP9, MMP2 and membrane type 1 metalloprotease MT1-MMP) [33], and their activator (EMMPRIN) [34]. Moreover, it has been shown that MVs derived from lipopolysaccharide-activated monocytes are able to induce apoptosis in target cells by transferring caspase-1 [35]. Therefore, MV-dependent transport of growth factors, receptors, anti- or pro-apoptotic or toxic molecules may support an interplay among heterogeneous cellular populations.

More recently, it has been shown that MVs may mediate a horizontal transfer of genetic information. Ratajczak *et al*. [3] demonstrated that MVs produced by murine embryonic stem cells may reprogramme haematopoietic progenitors by delivering not only proteins, but also mRNA for several pluripotent transcription factors that can be transferred to target cells, and subsequently

translated into proteins. The pretreatment of MVs with RNase inhibited the observed biological effects, indicating the important contribution of MV-derived mRNA [3].

Our group showed that MVs released from endothelial progenitor cells activate an angiogenic programme both *in vitro* and *in vivo*, by horizontal transfer of selected functional pro-angiogenic mRNA [24]. After the internalization of MVs into quiescent endothelial cells following interaction with α4 and β1 integrins expressed on the MV surface, the transferred mRNA was translated into the corresponding proteins. RNase pretreatment of MVs abrogated the angiogenic effect despite the MV internalization by endothelial cells, confirming the critical role for RNA transfer via MVs [24].

MSCs release a significant amount of MVs containing mRNA with specific multiple differentiative and functional properties, as well as selected patterns of mature microRNAs [26, 36]. These nucleic acids can be transferred via MVs to recipient cells, inducing functional and phenotypic changes. This observation engenders the possibility that stem cells may modulate their biological effects by delivering genetic information and altering the gene expression of target cells, through MV-mediated transfer of mRNA and microRNA. It has been shown that embryonic stem cell MVs contain a significant amount of microRNA and that a subset of this microRNA can be transferred to mouse embryonic fibroblasts *in vitro* [25]. In addition, it has been demonstrated that MVs released from human MSCs and human liver stem cells (HLSCs) contain ribonucleoproteins involved in the intracellular trafficking of RNA and selected patterns of microRNA, suggesting a dynamic regulation and compartmentalization of RNA in MVs produced by human adult stem cells of mesenchymal origin [26].

Therefore, MVs, by conveying selected patterns of proteins, lipids and nucleic acids to recipient cells, may be considered as being potent paracrine/endocrine factors involved in signalling between stem cells and differentiated cells (Figure 1). In addition, MVs contain subsets of RNA specific to the cell of origin. This is of particular relevance as exosomes released from mast cells exposed to oxidative stress communicate a protective message for reducing cell death to other cells exposed to oxidative stress, suggesting that the protective effect is partly mediated by the exosome-mediated transfer of RNA to target cells [37].

Stem cell-derived MVs may provoke epigenetic changes in target cells, e.g. haematopoietic progenitors [3], endothelial cells [24], kidney cells [38] and liver cells [39], inducing development regulation, regeneration and cell differentiation. Along with stem cells, adult stem cells that are present in all adult tissues also contribute to the processes involved in recovery after injury. Located in specific niches together with differentiated cells and tightly dependent on one another, stem and differentiated cells communicate with each other to regulate the self-renewal and differentiation processes related to tissue repair [40, 41]. In this context, the stem cell-derived MVs could participate in the reparative process [41]. Ratajczak *et al*. [3] demonstrated that embryonic stem cell-derived MVs increase the pluripotency of haematopoietic progenitors by transferring selectively enriched mRNA for certain transcription factors such as Oct-4, Rex-1, Nanog, stem cell leukaemia SCL and GATA-2 during their formation and, after fusion with target cells, the transferred mRNA is translated into protein by the recipient cells. Recently, Quesenberry *et al.* [42] and Aliotta *et al.* [43] suggested that MVs may be considered as an environmental signal for stem cell differentiation in the continuum model of stem cell biology. Murine lung-derived MVs enter into bone marrow cells and mediate lung-specific changes in the mRNA by direct delivery of mRNA and induction of transcription [43]. Therefore, stem cells and differentiated cells may establish bi-directional communication during the reparative process. Signals from injured cells may be critical to induce stem cell recruitment and stimulate their differentiation. In this context, MVs released from damaged tissues may organize adult resident stem cells into a reparative programme, mediated by paracrine effects. Indeed, MVs released from damaged tissues are enriched in mRNA specific for the injured tissue [44].

MVs derived from stem cells may reprogramme cells that survived injury and favour tissue regeneration

MVs could represent an important potential therapeutic tool. As they may influence the behaviour of recipient cells by delivering their bioactive cargo, this effect could be exploited in tissue regeneration and repair. In addition, the use of MVs instead of stem cells could represent a therapeutic strategy.

Bruno *et al*. [36] demonstrated that recovery from AKI after MSC administration may be mediated by the MVs released from MSCs and showed that human bone marrow MSC-derived MVs may activate a proliferative programme in tubular epithelial cells that survived injury both *in vitro* and in a glycerol-induced model of AKI in Severe Combined Immunodeficiency (SCID) mice. When compared with MSCs, MVs were found to mimic the beneficial effects of these cells, suggesting that they may mediate several of the regenerative functions of MSCs. The effects were RNA-dependent, since recovery from AKI was abolished by RNase pretreatment of MVs. MSC-MVs shuttled specific subsets of mRNA associated with the mesenchymal phenotype, responsible for controlling transcription, proliferation and immune regulation. These mRNA subsets were subsequently translated to proteins [36] and, along with soluble factors, are thought to contribute to the regenerative effect of MSCs. The effective transfer of specific microRNA and mRNA, and the translation of MV-shuttled mRNA into proteins within recipient cells were shown both *in vitro* and *in vivo* [26, 36]. MVs derived from MSCs were also shown to enhance survival in a cisplatin-induced lethal model of AKI in SCID mice [45]. In this model, the single administration of MVs ameliorated renal function and morphology and improved survival, but did not prevent chronic tubular injury and a persistent increase in BUN and creatinine. However, when mice were treated with

multiple injections of MVs, mortality further decreased, and at Day 21, the surviving mice showed normal histology and renal function. The mechanism of protection was mainly ascribed to an MV-induced up-regulation of anti-apoptotic genes in tubular epithelial cells, such as Bcl-xL, Bcl2 and baculoviral IAP repeat containing 8, along with down-regulation of genes with a central role in the execution phase of cell apoptosis such as Casp1, Casp8 and lymphotoxin alpha.

A similar protective effect of MSC-derived MVs was observed in a model of renal ischaemia/reperfusion injury. In this model, the administration of MVs not only limited acute injury by inhibiting apoptosis and stimulating proliferation, but also prevented the development of chronic renal disease [38].

In another study, Herrera *et al*. [39] demonstrated that following internalization, HLSC-derived MVs induced *in vitro* proliferation and apoptosis resistance of human and rat hepatocytes in an RNA-dependent manner; *in vivo* MVs accelerated the morphological and functional recovery of the liver in a model of 70% hepatectomy in rats, by means of proteins translated from MV-shuttled mRNA.

Timmers *et al*. [46] demonstrated a significant reduction in infarct size in pig and mouse models of ischaemia/reperfusion injury when CM from human embryonic stem cell-derived MSCs was intravenously administrated prior to reperfusion. It was later demonstrated by the same group that the cardioprotective effect was mediated by the release of exosomes from MSCs [47].

Conclusions

As suggested by Lai *et al*. [47], the paracrine hypothesis of MSC action has changed the perspective of the therapeutic use of MSCs in regenerative medicine. As MVs hold several biological properties of the cell of origin, the development of therapeutic strategies that avoid the administration of MSCs can be envisaged. This may attenuate many of the safety concerns relative to the use of living cells.

Based on clinical trials, MSC-based therapy is considered relatively safe, and to date, no relevant detrimental effects have been reported in humans. However, some concerns arise over the use of replicating cells that may escape from control over time [47]. Some potential complications could also arise from the intravascular administration of MSCs leading to vascular occlusion. Preclinical studies also do not exclude the possibility of maldifferentiation of injected MSCs. Indeed, myocardial calcification [48] and enhanced accumulation of fibroblasts and myofibroblasts in the lungs [49] have been reported following MSC treatment.

In an experimental model of glomerulonephritis, despite early beneficial effects, MSCs in the long-term were shown to maldifferentiate into adipocytes, leading to chronic renal injury [50].

MVs could also have certain advantages with respect to the use of soluble factors, the beneficial effects of which are limited by the difficulties of delivering them to the appropriate cell type [47]. In addition, the administration of a single factor cannot effectively mimic the actions of MSCs that concomitantly release a number of different factors. The delivery of MVs is driven by surface receptors that may be instrumental to the internalization within target cells. These receptors, which are shared with the membrane of MSCs, are also involved in MV recruitment at the site of injury. In addition, the cargo of MVs is protected by the physiological concentration of degrading enzymes present in plasma and tissues. Therefore, MVs may deliver a complex array of biologically active proteins and nucleic acids derived from MSCs to injured cells, which may favour tissue regeneration. Repeated administration of allogenic MVs derived from MSCs was shown not to elicit immune responses as they do not express histocompatibility antigens.

Several problems remain to be addressed before clinical use is considered. First, the issue of a large-scale production of MVs from cultured stem cells should be solved. Secondly, criteria for defining the potency of different MV preparations need to be clarified. Despite preliminary experiments in animals indicating the safety of MV administration, additional experiments are required to investigate long-term safety. Moreover, before MVs may be used in a clinical application, disease specificity, bio-distribution and persistency of the biologic effects must be validated.

In perspective, with the advances of cellular techniques, the engineering of the MVs surface or content, in order to enhance their disease specificity, may be envisaged.

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